

## SEQUENCE-DETERMINED DNA FRAGMENTS AND CORRESPONDING POLYPEPTIDES ENCODED THEREBY

This application claims priority under 35 USC §119(e), §119(a-d) and §120 of the following applications, the entire contents of which are hereby incorporated by reference:

Country	Attorney No.	Application No.	Filing Date
United States	2750-0785P	60/194,385	4/4/00
United States	2750-0789P	60/194,682	4/5/00
United States	2750-0792P	60/194,698	4/5/00
United States	2750-0784P	60/194,884	4/6/00
United States	2750-0797P	60/195,258	4/7/00
United States	2750-0802P	60/196,168	4/11/00
United States	2750-0805P	60/196,483	4/12/00
United States	2750-0814P	60/197,397	4/14/00
United States	2750-0817P	60/198,268	4/17/00
United States	2750-0820P	60/198,400	4/19/00
United States	2750-0823P	60/198,629	4/20/00
United States	2750-0826P	60/198,765	4/21/00
United States	2750-0829P	60/199,123	4/24/00

5

This application contains a CDR, the entire contents of which are hereby incorporated by reference. The CDR contains the following files:

File Name	Date of Creation	File Size
010214 Protein Domain Table	February 14, 2001	2.18 MB
2750-1434P Table 1	April 3, 2001	947 KB

### 10 FIELD OF THE INVENTION

The present invention relates to isolated polynucleotides that represent a complete gene, or a fragment thereof, that is expressed. In addition, the present invention relates to the polypeptide or protein corresponding to the coding sequence of these polynucleotides. The present invention also relates to isolated polynucleotides that represent regulatory regions of genes. The present invention also relates to isolated polynucleotides that represent untranslated regions of genes. The present invention further relates to the use of these isolated polynucleotides and polypeptides and proteins.

15

### DESCRIPTION OF THE RELATED ART

Efforts to map and sequence the genome of a number of organisms are in progress; a few complete genome sequences, for example those of *E. coli* and *Saccharomyces cerevisiae* are known (Blattner et al., *Science* 277:1453 (1997); Goffeau et al., *Science* 274:546 (1996)).

20

The complete genome of a multicellular organism, *C. elegans*, has also been sequenced (See, the *C. elegans* Sequencing Consortium, *Science* 282:2012 (1998)).

## 5 SUMMARY OF THE INVENTION

The present invention comprises polynucleotides, such as complete cDNA sequences and/or sequences of genomic DNA encompassing complete genes, fragments of genes, and/or regulatory elements of genes and/or regions with other functions and/or intergenic regions, hereinafter collectively referred to as Sequence-Determined DNA Fragments (SDFs), from 10 different plant species, particularly corn, wheat, soybean, rice and *Arabidopsis thaliana*, and other plants and or mutants, variants, fragments or fusions of said SDFs and polypeptides or proteins derived therefrom. In some instances, the SDFs span the entirety of a protein-coding segment. In some instances, the entirety of an mRNA is represented. Other objects of the invention that are also represented by SDFs of the invention are control sequences, such as, but 15 not limited to, promoters. Complements of any sequence of the invention are also considered part of the invention.

Other objects of the invention are polynucleotides comprising exon sequences, polynucleotides comprising intron sequences, polynucleotides comprising introns together with exons, intron/exon junction sequences, 5' untranslated sequences, and 3' untranslated 20 sequences of the SDFs of the present invention. Polynucleotides representing the joinder of any exons described herein, in any arrangement, for example, to produce a sequence encoding any desirable amino acid sequence are within the scope of the invention.

The present invention also resides in probes useful for isolating and identifying nucleic acids that hybridize to an SDF of the invention. The probes can be of any length, but more 25 typically are 12-2000 nucleotides in length; more typically, 15 to 200 nucleotides long; even more typically, 18 to 100 nucleotides long.

Yet another object of the invention is a method of isolating and/or identifying nucleic acids using the following steps:

- (a) contacting a probe of the instant invention with a polynucleotide sample under 30 conditions that permit hybridization and formation of a polynucleotide duplex; and
- (b) detecting and/or isolating the duplex of step (a).

The conditions for hybridization can be from low to moderate to high stringency conditions. The sample can include a polynucleotide having a sequence unique in a plant

genome. Probes and methods of the invention are useful, for example, without limitation, for mapping of genetic traits and/or for positional cloning of a desired fragment of genomic DNA.

Probes and methods of the invention can also be used for detecting alternatively spliced messages within a species. Probes and methods of the invention can further be used to detect 5 or isolate related genes in other plant species using genomic DNA (gDNA) and/or cDNA libraries. In some instances, especially when longer probes and low to moderate stringency hybridization conditions are used; the probe will hybridize to a plurality of cDNA and/or gDNA sequences of a plant. This approach is useful for isolating representatives of gene families which are identifiable by possession of a common functional domain in the gene 10 product or which have common cis-acting regulatory sequences. This approach is also useful for identifying orthologous genes from other organisms.

The present invention also resides in constructs for modulating the expression of the genes comprised of all or a fragment of an SDF. The constructs comprise all or a fragment of the expressed SDF, or of a complementary sequence. Examples of constructs include 15 ribozymes comprising RNA encoded by an SDF or by a sequence complementary thereto, antisense constructs, constructs comprising coding regions or parts thereof, constructs comprising promoters, introns, untranslated regions, scaffold attachment regions, methylating regions, enhancing or reducing regions, DNA and chromatin conformation modifying sequences, etc. Such constructs can be constructed using viral, plasmid, bacterial 20 artificial chromosomes (BACs), plasmid artificial chromosomes (PACs), autonomous plant plasmids, plant artificial chromosomes or other types of vectors and exist in the plant as autonomous replicating sequences or as DNA integrated into the genome. When inserted into a host cell the construct is, preferably, functionally integrated with, or operatively linked to, a heterologous polynucleotide. For instance, a coding region from an SDF might 25 be operably linked to a promoter that is functional in a plant.

The present invention also resides in host cells, including bacterial or yeast cells or plant cells, and plants that harbor constructs such as described above. Another aspect of the invention relates to methods for modulating expression of specific genes in plants by expression of the coding sequence of the constructs, by regulation of expression of one or more 30 endogenous genes in a plant or by suppression of expression of the polynucleotides of the invention in a plant. Methods of modulation of gene expression include without limitation (1) inserting into a host cell additional copies of a polynucleotide comprising a coding sequence; (2) modulating an endogenous promoter in a host cell; (3) inserting antisense or ribozyme constructs into a host cell and (4) inserting into a host cell a polynucleotide comprising a

sequence encoding a variant, fragment, or fusion of the native polypeptides of the instant invention.

## BRIEF DESCRIPTION OF THE TABLES

5 In TABLE 1, the format of the data is as follows:

TABLE 1, in general, is divided into a plurality of sections, with each section presenting a number of genes described by coding sequences which, in combination, define each gene, followed by the amino acid sequence coded for by each gene. Each section of TABLE 1 begins with a line labeled "Seq name", followed by the sequence identifier "gi" 10 and a unique number which identifies a specific DNA sequence in the publicly accessible BLAST Databases on the NCBI FTP web site (accessible at ncbi.nlm.gov/blast). In particular, the "nt.Z" nucleotide sequence data base at the NCBI FTP site utilizes the "gi" 15 identifiers to assign by NCBI a unique identifier for each sequence in the databases, thereby providing a non-redundant database for sequences from various data bases, including GenBank, EMBL, DDBJ (DNA Database of Japan) and PDB (Brookhaven Protein Data Bank). Thus, the line in TABLE 1 beginning with "Seq name" identifies the unique "gi" identifier.

The next line in TABLE 1 describes the number of base pairs (bp) and percentage G+C content in that identified public data base DNA sequence. The next two lines in 20 TABLE 1 identify the number of genes and exons in the (+) chain/strand and the (-) chain/strand of that public data base sequence identified as the present invention. Each section of TABLE 1 then includes the following information in columns identified as:

"G" identifies the gene number in the DNA sequence that is identified by the unique "gi" number.

25 "Str" identifies whether the sequence is from the plus (+) or minus (-) strand of the DNA.

"Feature" identifies whether the sequence is a polyadenylation signal (Pol A), a first coding sequence ("CDSf"), an internal coding sequence ("CDSi"), a last coding sequence ("CDSl"), the only coding sequence (CDSo) or a transcriptional start sequence 30 ("TSS").

"Start" and "End" identify the bases in the "gi" DNA sequence which are the beginning and end (inclusive) of the corresponding portion identified under the "Type" column.

“ORF” identifies the bases in the “gi” DNA sequence which form the corresponding open reading frame.

“Len” identifies the length in number of nucleotides of the identified coding portion of the gene.

5 Following this tabulated information of the DNA sequences (SDFs of the present invention), TABLE 1 presents a plurality of corresponding amino acid sequences coded by each SDF. The information line preceding each amino acid sequence identifies the gene number (e.g. “ARAGEN 1”), the number of exons which comprise the gene, the first and the last base number for the coding region, the total number of amino acids, and whether the 10 amino acid sequence is coded by the + or - chain/strand of the gene.

In TABLE 2, if present, the format of the data is as follows:

15 TABLE 2, in general, is divided into a plurality of sections, with each section presenting a number of sequences in the publicly accessible BLAST Databases on the NCBI FTP web site found to have a high degree of amino acid sequence homology to the predicted proteins in Table 1. Each section of TABLE 2 begins with a number corresponding to the unique “gi” number following “Seq name” in the first line of each section of Table 1. The “gi” number is connected with a dash to a “gn” number (the two combined numbers separated by a dash are hereafter referred to as the “gi-gn” number). The “gn” number corresponds to each gene number within the section of Table 1 identified by 20 the “gi” number. Following each “gi-gn” number is a list of sequences in the publicly accessible BLAST Databases found to have a high degree of amino acid sequence homology to the amino acid sequence given in Table 1 and identified by the “gi-gn” number. Some “gi-gn” numbers may not have any sequences in the publicly accessible BLAST Databases with a high degree of amino acid sequence homology.

25 Each sequence in the list is identified with a unique “gi” identifier followed by a corresponding database identifier, accession number, and a locus description. The locus descriptions are truncated in the table, however the full locus descriptions can be located by the “gi” number and viewed in the publicly accessible database. Following the locus description is a number indicating the degree of reliability of sequence identity, with a 30 higher number indicating a higher likelihood that the sequence in the public database matches the corresponding amino acid sequence predicted in Table 1. The next number is a P value, indicating the probability of finding the sequence by chance. The numbers are given in exponent form. For example “4.9e-16”, is equivalent to  $4.9 \times 10^{-16}$ . “0.0e+00” indicates a zero percent probability of finding the sequence by chance. Following the P

value is a number indicating how many segments in the indicated sequence match the corresponding amino acid sequence predicted in Table 1. A number greater than one indicates that multiple segments match, and each matching segment is separated from the other segments by gaps of sequence that do not match. The number "1" indicates that the 5 public sequence and the sequence predicted in Table 1 share sequence homology over one continuous stretch of sequence.

## DETAILED DESCRIPTION OF THE INVENTION

The invention relates to (I) polynucleotides and methods of use thereof, such as

- IA. Probes, Primers and Substrates;
- IB. Methods of Detection and Isolation;
  - B.1. Hybridization;
  - B.2. Methods of Mapping;
  - B.3. Southern Blotting;
  - B.4. Isolating cDNA from Related Organisms;
  - B.5. Isolating and/or Identifying Orthologous Genes
- IC. Methods of Inhibiting Gene Expression
  - C.1. Antisense
  - C.2. Ribozyme Constructs;
  - C.3. Chimeraplasts;
  - C.4. Co-Suppression;
  - C.5. Transcriptional Silencing
  - C.6. Other Methods to Inhibit Gene Expression
- ID. Methods of Functional Analysis;
- IE. Promoter Sequences and Their Use;
- IF. UTRs and/or Intron Sequences and Their Use; and
- IG. Coding Sequences and Their Use.

The invention also relates to (II) polypeptides and proteins and methods of use thereof,

such as

- IIA. Native Polypeptides and Proteins
  - A.1 Antibodies
  - A.2 In Vitro Applications
- IIB. Polypeptide Variants, Fragments and Fusions
  - B.1 Variants

B.2 Fragments

B.3 Fusions

The invention also includes (III) methods of modulating polypeptide production, such

5 as

IIIA. Suppression

A.1 Antisense

A.2 Ribozymes

A.3 Co-suppression

10 A.4 Insertion of Sequences into the Gene to be Modulated

A.5 Promoter Modulation

A.6 Expression of Genes containing Dominant-Negative Mutations

IIIB. Enhanced Expression

B.1 Insertion of an Exogenous Gene

15 B.2 Promoter Modulation

The invention further concerns (IV) gene constructs and vector construction, such as

IVA. Coding Sequences

IVB. Promoters

20 IVC. Signal Peptides

The invention still further relates to

V Transformation Techniques

25

**Definitions**

Allelic variant An "allelic variant" is an alternative form of the same SDF, which resides at the same chromosomal locus in the organism. Allelic variations can occur in any portion of the gene sequence, including regulatory regions. Allelic variants can arise by normal genetic variation in a population. Allelic variants can also be produced by genetic

30

engineering methods. An allelic variant can be one that is found in a naturally occurring plant, including a cultivar or ecotype. An allelic variant may or may not give rise to a phenotypic change, and may or may not be expressed. An allele can result in a detectable change in the phenotype of the trait represented by the locus. A phenotypically silent allele can give rise to a product.

5 Alternatively spliced messages Within the context of the current invention, “alternatively spliced messages” refers to mature mRNAs originating from a single gene with variations in the number and/or identity of exons, introns and/or intron-exon junctions.

10 Chimeric The term “chimeric” is used to describe genes, as defined supra, or constructs wherein at least two of the elements of the gene or construct, such as the promoter and the coding sequence and/or other regulatory sequences and/or filler sequences and/or complements thereof, are heterologous to each other.

15 Constitutive Promoter: Promoters referred to herein as "constitutive promoters" actively promote transcription under most, but not necessarily all, environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include the cauliflower mosaic virus (CaMV) 35S transcript initiation region and the 1' or 2' promoter 20 derived from T-DNA of *Agrobacterium tumefaciens*, and other transcription initiation regions from various plant genes, such as the maize ubiquitin-1 promoter, known to those of skill.

25 Coordinately Expressed: The term “coordinately expressed,” as used in the current invention, refers to genes that are expressed at the same or a similar time and/or stage and/or under the same or similar environmental conditions.

30 Domain: Domains are fingerprints or signatures that can be used to characterize protein families and/or parts of proteins. Such fingerprints or signatures can comprise conserved (1) primary sequence, (2) secondary structure, and/or (3) three-dimensional conformation. Generally, each domain has been associated with either a family of proteins or motifs. Typically, these families and/or motifs have been correlated with specific *in-vitro* and/or *in-vivo* activities. A domain can be any length, including the entirety of the sequence of a protein. Detailed descriptions of the domains, associated families and motifs, and correlated activities of the polypeptides of the instant invention are described below.

Usually, the polypeptides with designated domain(s) can exhibit at least one activity that is exhibited by any polypeptide that comprises the same domain(s).

5 Endogenous The term “endogenous,” within the context of the current invention refers to any polynucleotide, polypeptide or protein sequence which is a natural part of a cell or organisms regenerated from said cell.

Exogenous “Exogenous,” as referred to within, is any polynucleotide, 10 polypeptide or protein sequence, whether chimeric or not, that is initially or subsequently introduced into the genome of an individual host cell or the organism regenerated from said host cell by any means other than by a sexual cross. Examples of means by which this can be accomplished are described below, and include *Agrobacterium*-mediated transformation (of dicots - *e.g.* Salomon et al. *EMBO J.* 3:141 (1984); Herrera-Estrella et al. *EMBO J.* 15 2:987 (1983); of monocots, representative papers are those by Escudero et al., *Plant J.* 10:355 (1996), Ishida et al., *Nature Biotechnology* 14:745 (1996), May et al., *Bio/Technology* 13:486 (1995)), biolistic methods (Armaleo et al., *Current Genetics* 17:97 1990)), electroporation, *in planta* techniques, and the like. Such a plant containing the exogenous nucleic acid is referred to here as a T<sub>0</sub> for the primary transgenic plant and T<sub>1</sub> for 20 the first generation. The term “exogenous” as used herein is also intended to encompass inserting a naturally found element into a non-naturally found location.

Filler sequence: As used herein, “filler sequence” refers to any nucleotide sequence 25 that is inserted into DNA construct to evoke a particular spacing between particular components such as a promoter and a coding region and may provide an additional attribute such as a restriction enzyme site.

Gene: The term “gene,” as used in the context of the current invention, encompasses all regulatory and coding sequence contiguously associated with a single hereditary unit with a 30 genetic function (see SCHEMATIC 1). Genes can include non-coding sequences that modulate the genetic function that include, but are not limited to, those that specify polyadenylation, transcriptional regulation, DNA conformation, chromatin conformation, extent and position of base methylation and binding sites of proteins that control all of these. Genes comprised of “exons” (coding sequences), which may be interrupted by

10

“introns” (non-coding sequences), encode proteins. A gene’s genetic function may require only RNA expression or protein production, or may only require binding of proteins and/or nucleic acids without associated expression. In certain cases, genes adjacent to one another may share sequence in such a way that one gene will overlap the other. A gene can be found within the genome of an organism, artificial chromosome, plasmid, vector, etc., or as a separate isolated entity.

Gene Family: “Gene family” is used in the current invention to describe a group of functionally related genes, each of which encodes a separate protein.

10

Heterologous sequences: “Heterologous sequences” are those that are not operatively linked or are not contiguous to each other in nature. For example, a promoter from corn is considered heterologous to an *Arabidopsis* coding region sequence. Also, a promoter from a gene encoding a growth factor from corn is considered heterologous to a sequence encoding the corn receptor for the growth factor. Regulatory element sequences, such as UTRs or 3’ end termination sequences that do not originate in nature from the same gene as the coding sequence originates from, are considered heterologous to said coding sequence. Elements operatively linked in nature and contiguous to each other are not heterologous to each other. On the other hand, these same elements remain operatively linked but become heterologous if other filler sequence is placed between them. Thus, the promoter and coding sequences of a corn gene expressing an amino acid transporter are not heterologous to each other, but the promoter and coding sequence of a corn gene operatively linked in a novel manner are heterologous.

15

20 if other filler sequence is placed between them. Thus, the promoter and coding sequences of a corn gene expressing an amino acid transporter are not heterologous to each other, but the promoter and coding sequence of a corn gene operatively linked in a novel manner are heterologous.

25

Homologous gene In the current invention, “homologous gene” refers to a gene that shares sequence similarity with the gene of interest. This similarity may be in only a fragment of the sequence and often represents a functional domain such as, examples including without limitation a DNA binding domain, a domain with tyrosine kinase activity, or the like. The functional activities of homologous genes are not necessarily the same.

30

Inducible Promoter An “inducible promoter” in the context of the current invention refers to a promoter which is regulated under certain conditions, such as light, chemical concentration, protein concentration, conditions in an organism, cell, or organelle, etc. A typical example of an inducible promoter, which can be utilized with the polynucleotides of the

present invention, is PARSK1, the promoter from the *Arabidopsis* gene encoding a serine-threonine kinase enzyme, and which promoter is induced by dehydration, abscissic acid and sodium chloride (Wang and Goodman, *Plant J.* 8:37 (1995)) Examples of environmental conditions that may affect transcription by inducible promoters include anaerobic conditions, elevated temperature, or the presence of light.

5

Intergenic region "Intergenic region," as used in the current invention, refers to nucleotide sequence occurring in the genome that separates adjacent genes.

10

Mutant gene In the current invention, "mutant" refers to a heritable change in DNA sequence at a specific location. Mutants of the current invention may or may not have an associated identifiable function when the mutant gene is transcribed.

15

Orthologous Gene In the current invention "orthologous gene" refers to a second gene that encodes a gene product that performs a similar function as the product of a first gene. The orthologous gene may also have a degree of sequence similarity to the first gene. The orthologous gene may encode a polypeptide that exhibits a degree of sequence similarity to a polypeptide corresponding to a first gene. The sequence similarity can be found within a functional domain or along the entire length of the coding sequence of the genes and/or their corresponding polypeptides.

20

Percentage of sequence identity "Percentage of sequence identity," as used herein, is determined by comparing two optimally aligned sequences over a comparison window, where the fragment of the polynucleotide or amino acid sequence in the comparison window may comprise additions or deletions (e.g., gaps or overhangs) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman *Add. APL. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch *J. Mol. Biol.* 48:443

25

30

(1970), by the search for similarity method of Pearson and Lipman *Proc. Natl. Acad. Sci. (USA)* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, BLAST, PASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by inspection. Given 5 that two sequences have been identified for comparison, GAP and BESTFIT are preferably employed to determine their optimal alignment. Typically, the default values of 5.00 for gap weight and 0.30 for gap weight length are used. The term "substantial sequence identity" between polynucleotide or polypeptide sequences refers to polynucleotide or polypeptide comprising a sequence that has at least 80% sequence identity, preferably at least 85%, more 10 preferably at least 90% and most preferably at least 95%, even more preferably, at least 96%, 97%, 98% or 99% sequence identity compared to a reference sequence using the programs.

Plant Promoter        A "plant promoter" is a promoter capable of initiating transcription in plant cells and can drive or facilitate transcription of a fragment of the SDF of the instant 15 invention or a coding sequence of the SDF of the instant invention. Such promoters need not be of plant origin. For example, promoters derived from plant viruses, such as the CaMV35S promoter or from *Agrobacterium tumefaciens* such as the T-DNA promoters, can be plant promoters. A typical example of a plant promoter of plant origin is the maize ubiquitin-1 (ubi-1) promoter known to those of skill.

20

Promoter:        The term "promoter," as used herein, refers to a region of sequence determinants located upstream from the start of transcription of a gene and which are involved 25 in recognition and binding of RNA polymerase and other proteins to initiate and modulate transcription. A basal promoter is the minimal sequence necessary for assembly of a transcription complex required for transcription initiation. Basal promoters frequently include a "TATA box" element usually located between 15 and 35 nucleotides upstream from the site of initiation of transcription. Basal promoters also sometimes include a "CCAAT box" 30 element (typically a sequence CCAAT) and/or a GGGCG sequence, usually located between 40 and 200 nucleotides, preferably 60 to 120 nucleotides, upstream from the start site of transcription.

Public sequence: The term “public sequence ,” as used in the context of the instant application, refers to any sequence that has been deposited in a publicly accessible database. This term encompasses both amino acid and nucleotide sequences. Such sequences are publicly accessible, for example, on the BLAST databases on the NCBI FTP web site (accessible at ncbi.nlm.gov/blast). The database at the NCBI GTP site utilizes “gi” numbers assigned by NCBI as a unique identifier for each sequence in the databases, thereby providing a non-redundant database for sequence from various databases, including GenBank, EMBL, DBBJ, (DNA Database of Japan) and PDB (Brookhaven Protein Data Bank).

10

Regulatory Sequence The term “regulatory sequence,” as used in the current invention, refers to any nucleotide sequence that influences transcription or translation initiation and rate, and stability and/or mobility of the transcript or polypeptide product. Regulatory sequences include, but are not limited to, promoters, promoter control elements, protein binding sequences, 5' and 3' UTRs, transcriptional start site, termination sequence, polyadenylation sequence, introns, certain sequences within a coding sequence, etc.

15  
20  
25  
30

Related Sequences: “Related sequences” refer to either a polypeptide or a nucleotide sequence that exhibits some degree of sequence similarity with a sequence described in Table 1 and Table 2, if present.

Scaffold Attachment Region (SAR) As used herein, “scaffold attachment region” is a DNA sequence that anchors chromatin to the nuclear matrix or scaffold to generate loop domains that can have either a transcriptionally active or inactive structure (Spiker and Thompson 1996) Plant Physiol. 110: 15-21).

Sequence-determined DNA fragments (SDFs) “Sequence-determined DNA fragments” as used in the current invention are isolated sequences of genes, fragments of genes, intergenic regions or contiguous DNA from plant genomic DNA or cDNA or RNA the sequence of which has been determined.

Signal Peptide A “signal peptide” as used in the current invention is an amino acid sequence that targets the protein for secretion, for transport to an intracellular compartment

or organelle or for incorporation into a membrane. Signal peptides are indicated in the tables and a more detailed description located below.

Specific Promoter In the context of the current invention, "specific promoters" refers to a subset of inducible promoters that have a high preference for being induced in a specific tissue or cell and/or at a specific time during development of an organism. By "high preference" is meant at least 3-fold, preferably 5-fold, more preferably at least 10-fold still more preferably at least 20-fold, 50-fold or 100-fold increase in transcription in the desired tissue over the transcription in any other tissue. Typical examples of temporal and/or tissue specific promoters of plant origin that can be used with the polynucleotides of the present invention, are: PTA29, a promoter which is capable of driving gene transcription specifically in tapetum and only during anther development (Koltonow et al., *Plant Cell* 2:1201 (1990); RCc2 and RCc3, promoters that direct root-specific gene transcription in rice (Xu et al., *Plant Mol. Biol.* 27:237 (1995); TobRB27, a root-specific promoter from tobacco (Yamamoto et al., *Plant Cell* 3:371 (1991)). Examples of tissue-specific promoters under developmental control include promoters that initiate transcription only in certain tissues or organs, such as root, ovule, fruit, seeds, or flowers. Other suitable promoters include those from genes encoding storage proteins or the lipid body membrane protein, oleosin. A few root-specific promoters are noted above.

Stringency "Stringency" as used herein is a function of probe length, probe composition (G + C content), and salt concentration, organic solvent concentration, and temperature of hybridization or wash conditions. Stringency is typically compared by the parameter  $T_m$ , which is the temperature at which 50% of the complementary molecules in the hybridization are hybridized, in terms of a temperature differential from  $T_m$ . High stringency conditions are those providing a condition of  $T_m - 5^\circ\text{C}$  to  $T_m - 10^\circ\text{C}$ . Medium or moderate stringency conditions are those providing  $T_m - 20^\circ\text{C}$  to  $T_m - 29^\circ\text{C}$ . Low stringency conditions are those providing a condition of  $T_m - 40^\circ\text{C}$  to  $T_m - 48^\circ\text{C}$ . The relationship of hybridization conditions to  $T_m$  (in  $^\circ\text{C}$ ) is expressed in the mathematical equation

$$30 \quad T_m = 81.5 - 16.6(\log_{10}[\text{Na}^+]) + 0.41(\%G+C) - (600/N) \quad (1)$$

where N is the length of the probe. This equation works well for probes 14 to 70 nucleotides in length that are identical to the target sequence. The equation below for  $T_m$  of DNA-DNA

hybrids is useful for probes in the range of 50 to greater than 500 nucleotides, and for conditions that include an organic solvent (formamide).

$$T_m = 81.5 + 16.6 \log \{ [Na^+] / (1 + 0.7[Na^+]) \} + 0.41(\%G+C) - 500/L - 0.63(\%formamide) \quad (2)$$

where L is the length of the probe in the hybrid. (P. Tijessen, "Hybridization with Nucleic

5 Acid Probes" in Laboratory Techniques in Biochemistry and Molecular Biology, P.C. vander Vliet, ed., c. 1993 by Elsevier, Amsterdam.) The  $T_m$  of equation (2) is affected by the nature of the hybrid; for DNA-RNA hybrids  $T_m$  is 10-15°C higher than calculated, for RNA-RNA hybrids  $T_m$  is 20-25°C higher. Because the  $T_m$  decreases about 1 °C for each 1% decrease in homology when a long probe is used (Bonner et al., *J. Mol. Biol.* 81:123 (1973)),  
10 stringency conditions can be adjusted to favor detection of identical genes or related family members.

Equation (2) is derived assuming equilibrium and therefore, hybridizations according to the present invention are most preferably performed under conditions of probe excess and for sufficient time to achieve equilibrium. The time required to reach equilibrium can be shortened by inclusion of a hybridization accelerator such as dextran sulfate or another high volume polymer in the hybridization buffer.

15 Stringency can be controlled during the hybridization reaction or after hybridization has occurred by altering the salt and temperature conditions of the wash solutions used. The formulas shown above are equally valid when used to compute the stringency of a wash solution. Preferred wash solution stringencies lie within the ranges stated above; high stringency is 5-8°C below  $T_m$ , medium or moderate stringency is 26-29°C below  $T_m$  and low stringency is 45-48°C below  $T_m$ .

20 Substantially free of      A composition containing A is "substantially free of" B when at least 85% by weight of the total A+B in the composition is A. Preferably, A comprises at least about 90% by weight of the total of A+B in the composition, more preferably at least about 95% or even 99% by weight. For example, a plant gene or DNA sequence can be considered substantially free of other plant genes or DNA sequences.

25 Translational start site      In the context of the current invention, a "translational start site" is usually an ATG in the cDNA transcript, more usually the first ATG. A single cDNA, however, may have multiple translational start sites.

Transcription start site        “Transcription start site” is used in the current invention to describe the point at which transcription is initiated. This point is typically located about 25 nucleotides downstream from a TFIID binding site, such as a TATA box. Transcription can 5 initiate at one or more sites within the gene, and a single gene may have multiple transcriptional start sites, some of which may be specific for transcription in a particular cell-type or tissue.

Untranslated region (UTR)    A “UTR” is any contiguous series of nucleotide bases that is 10 transcribed, but is not translated. These untranslated regions may be associated with particular functions such as increasing mRNA message stability. Examples of UTRs include, but are not limited to polyadenylation signals, terminations sequences, sequences located between the transcriptional start site and the first exon (5’ UTR) and sequences located between the last exon and the end of the mRNA (3’ UTR).

15        Variant:        The term “variant” is used herein to denote a polypeptide or protein or polynucleotide molecule that differs from others of its kind in some way. For example, polypeptide and protein variants can consist of changes in amino acid sequence and/or charge and/or post-translational modifications (such as glycosylation, etc).

## DETAILED DESCRIPTION OF THE INVENTION

### I. Polynucleotides

Exemplified SDFs of the invention represent fragments of the genome of corn, wheat, 25 rice, soybean or *Arabidopsis* and/or represent mRNA expressed from that genome. The isolated nucleic acid of the invention also encompasses corresponding fragments of the genome and/or cDNA complement of other organisms as described in detail below.

Polynucleotides of the invention can be isolated from polynucleotide libraries using primers comprising sequence similar to those described by Table 1 and Table 2, if present. 30 See, for example, the methods described in Sambrook et al., *supra*.

Alternatively, the polynucleotides of the invention can be produced by chemical synthesis. Such synthesis methods are described below.

5

It is contemplated that the nucleotide sequences presented herein may contain some small percentage of errors. These errors may arise in the normal course of determination of nucleotide sequences. Sequence errors can be corrected by obtaining seeds deposited under the accession numbers cited above, propagating them, isolating genomic DNA or appropriate 10 mRNA from the resulting plants or seeds thereof, amplifying the relevant fragment of the genomic DNA or mRNA using primers having a sequence that flanks the erroneous sequence, and sequencing the amplification product.

#### I.A. Probes, Primers and Substrates

SDFs of the invention can be applied to substrates for use in array applications such 15 as, but not limited to, assays of global gene expression, for example under varying conditions of development, growth conditions. The arrays can also be used in diagnostic or forensic methods (WO95/35505, US 5,445,943 and US 5,410,270).

Probes and primers of the instant invention will hybridize to a polynucleotide comprising a sequence in Table 1 and Table 2, if present. Though many different 20 nucleotide sequences can encode an amino acid sequence, the sequences of Table 1 and Table 2, if present are generally preferred for encoding polypeptides of the invention. However, the sequence of the probes and/or primers of the instant invention need not be identical to those in Table 1 and Table 2, if present or the complements thereof. For example, some variation in probe or primer sequence and/or length can allow additional 25 family members to be detected, as well as orthologous genes and more taxonomically distant related sequences. Similarly, probes and/or primers of the invention can include additional nucleotides that serve as a label for detecting the formed duplex or for subsequent cloning purposes.

Probe length will vary depending on the application. For use as primers, probes are 30 12-40 nucleotides, preferably 18-30 nucleotides long. For use in mapping, probes are preferably 50 to 500 nucleotides, preferably 100-250 nucleotides long. For Southern hybridizations, probes as long as several kilobases can be used as explained below.

The probes and/or primers can be produced by synthetic procedures such as the triester method of Matteucci et al. *J. Am. Chem. Soc.* 103:3185( 1981); or according to Urdea et al. *Proc. Natl. Acad.* 80:7461 (1981) or using commercially available automated oligonucleotide synthesizers.

5

### I.B. Methods of Detection and Isolation

The polynucleotides of the invention can be utilized in a number of methods known to those skilled in the art as probes and/or primers to isolate and detect polynucleotides, including, without limitation: Southernns, Northerns, Branched DNA hybridization assays, polymerase chain reaction, and microarray assays, and variations thereof. Specific methods given by way of examples, and discussed below include:

Hybridization

Methods of Mapping

15 Southern Blotting

Isolating cDNA from Related Organisms

Isolating and/or Identifying Orthologous Genes.

Also, the nucleic acid molecules of the invention can used in other methods, such as high density oligonucleotide hybridizing assays, described, for example, in U.S. Pat. Nos.

20 6,004,753; 5,945,306; 5,945,287; 5,945,308; 5,919,686; 5,919,661; 5,919,627; 5,874,248; 5,871,973; 5,871,971; and 5,871,930; and PCT Pub. Nos. WO 9946380; WO 9933981; WO 9933870; WO 9931252; WO 9915658; WO 9906572; WO 9858052; WO 9958672; and WO 9810858.

25 B.1. Hybridization

The isolated SDFs of Table 1 and Table 2, if present of the present invention can be used as probes and/or primers for detection and/or isolation of related polynucleotide sequences through hybridization. Hybridization of one nucleic acid to another constitutes a physical property that defines the subject SDF of the invention and the identified related sequences. Also, such hybridization imposes structural limitations on the pair. A good general discussion of the factors for determining hybridization conditions is provided by 30 Sambrook et al. ("Molecular Cloning, a Laboratory Manual, 2nd ed., c. 1989 by Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; *see esp.*, chapters 11 and 12). Additional considerations and details of the physical chemistry of hybridization are provided by G.H.

19

Keller and M.M. Manak "DNA Probes", 2<sup>nd</sup> Ed. pp. 1-25, c. 1993 by Stockton Press, New York, NY.

Depending on the stringency of the conditions under which these probes and/or primers are used, polynucleotides exhibiting a wide range of similarity to those in Table 1 and Table 2, if present can be detected or isolated. When the practitioner wishes to examine the result of membrane hybridizations under a variety of stringencies, an efficient way to do so is to perform the hybridization under a low stringency condition, then to wash the hybridization membrane under increasingly stringent conditions.

When using SDFs to identify orthologous genes in other species, the practitioner will preferably adjust the amount of target DNA of each species so that, as nearly as is practical, the same number of genome equivalents are present for each species examined. This prevents faint signals from species having large genomes, and thus small numbers of genome equivalents per mass of DNA, from erroneously being interpreted as absence of the corresponding gene in the genome.

The probes and/or primers of the instant invention can also be used to detect or isolate nucleotides that are "identical" to the probes or primers. Two nucleic acid sequences or polypeptides are said to be "identical" if the sequence of nucleotides or amino acid residues, respectively, in the two sequences is the same when aligned for maximum correspondence as described below.

Isolated polynucleotides within the scope of the invention also include allelic variants of the specific sequences presented in Table 1 and Table 2, if present. The probes and/or primers of the invention can also be used to detect and/or isolate polynucleotides exhibiting at least 80% sequence identity with the sequences of Table 1 and Table 2, if present or fragments thereof.

With respect to nucleotide sequences, degeneracy of the genetic code provides the possibility to substitute at least one base of the base sequence of a gene with a different base without causing the amino acid sequence of the polypeptide produced from the gene to be changed. Hence, the DNA of the present invention may also have any base sequence that has been changed from a sequence in Table 1 and Table 2, if present by substitution in accordance with degeneracy of genetic code. References describing codon usage include: Carels *et al.*, *J. Mol. Evol.* 46: 45 (1998) and Fennoy *et al.*, *Nucl. Acids Res.* 21(23): 5294 (1993).

B.2. Mapping

The isolated SDF DNA of the invention can be used to create various types of genetic and physical maps of the genome of corn, *Arabidopsis*, soybean, rice, wheat, or other plants. Some SDFs may be absolutely associated with particular phenotypic traits, 5 allowing construction of gross genetic maps. While not all SDFs will immediately be associated with a phenotype, all SDFs can be used as probes for identifying polymorphisms associated with phenotypes of interest. Briefly, one method of mapping involves total DNA isolation from individuals. It is subsequently cleaved with one or more restriction enzymes, separated according to mass, transferred to a solid support, hybridized with SDF DNA and 10 the pattern of fragments compared. Polymorphisms associated with a particular SDF are visualized as differences in the size of fragments produced between individual DNA samples after digestion with a particular restriction enzyme and hybridization with the SDF. After identification of polymorphic SDF sequences, linkage studies can be conducted. By using the individuals showing polymorphisms as parents in crossing programs, F2 progeny 15 recombinants or recombinant inbreds, for example, are then analyzed. The order of DNA polymorphisms along the chromosomes can be determined based on the frequency with which they are inherited together versus independently. The closer two polymorphisms are together in a chromosome the higher the probability that they are inherited together. Integration of the relative positions of all the polymorphisms and associated marker SDFs 20 can produce a genetic map of the species, where the distances between markers reflect the recombination frequencies in that chromosome segment.

The use of recombinant inbred lines for such genetic mapping is described for *Arabidopsis* by Alonso-Blanco et al. (*Methods in Molecular Biology*, vol.82, “*Arabidopsis Protocols*”, pp. 137-146, J.M. Martinez-Zapater and J. Salinas, eds., c. 1998 by Humana Press, Totowa, NJ) and for corn by Burr (“Mapping Genes with Recombinant Inbreds”, pp. 249-254. In Freeling, M. and V. Walbot (Ed.), *The Maize Handbook*, c. 1994 by Springer-Verlag New York, Inc.: New York, NY, USA; Berlin Germany; Burr et al. *Genetics* (1998) 118: 519; Gardiner, J. et al., (1993) *Genetics* 134: 917). This procedure, however, is not limited to plants and can be used for other organisms (such as yeast) or for individual cells.

30 The SDFs of the present invention can also be used for simple sequence repeat (SSR) mapping. Rice SSR mapping is described by Morgante et al. (*The Plant Journal* (1993) 3: 165), Panaud et al. (*Genome* (1995) 38: 1170); Senior et al. (*Crop Science* (1996) 36: 1676), Taramino et al. (*Genome* (1996) 39: 277) and Ahn et al. (*Molecular and General*

Genetics (1993) 241: 483-90). SSR mapping can be achieved using various methods. In one instance, polymorphisms are identified when sequence specific probes contained within an SDF flanking an SSR are made and used in polymerase chain reaction (PCR) assays with template DNA from two or more individuals of interest. Here, a change in the number of tandem repeats between the SSR-flanking sequences produces differently sized fragments (U.S. Patent 5,766,847). Alternatively, polymorphisms can be identified by using the PCR fragment produced from the SSR-flanking sequence specific primer reaction as a probe against Southern blots representing different individuals (U.H. Refseth et al., (1997) *Electrophoresis* 18: 1519).

Genetic and physical maps of crop species have many uses. For example, these maps can be used to devise positional cloning strategies for isolating novel genes from the mapped crop species. In addition, because the genomes of closely related species are largely syntenic (that is, they display the same ordering of genes within the genome), these maps can be used to isolate novel alleles from relatives of crop species by positional cloning strategies.

The various types of maps discussed above can be used with the SDFs of the invention to identify Quantitative Trait Loci (QTLs). Many important crop traits, such as the solids content of tomatoes, are quantitative traits and result from the combined interactions of several genes. These genes reside at different loci in the genome, oftentimes on different chromosomes, and generally exhibit multiple alleles at each locus. The SDFs of the invention can be used to identify QTLs and isolate specific alleles as described by de Vicente and Tanksley (*Genetics* 134:585 (1993)). In addition to isolating QTL alleles in present crop species, the SDFs of the invention can also be used to isolate alleles from the corresponding QTL of wild relatives. Transgenic plants having various combinations of QTL alleles can then be created and the effects of the combinations measured. Once a desired allele combination has been identified, crop improvement can be accomplished either through biotechnological means or by directed conventional breeding programs (for review see Tanksley and McCouch, *Science* 277:1063 (1997)).

In another embodiment, the SDFs can be used to help create physical maps of the genome of corn, *Arabidopsis* and related species. Where SDFs have been ordered on a genetic map, as described above, they can be used as probes to discover which clones in large libraries of plant DNA fragments in YACs, BACs, etc. contain the same SDF or similar sequences, thereby facilitating the assignment of the large DNA fragments to chromosomal positions. Subsequently, the large BACs, YACs, etc. can be ordered

unambiguously by more detailed studies of their sequence composition (e.g. Marra et al. (1997) Genomic Research 7:1072-1084) and by using their end or other sequences to find the identical sequences in other cloned DNA fragments. The overlapping of DNA sequences in this way allows large contigs of plant sequences to be built that, when sufficiently extended, provide a complete physical map of a chromosome. Sometimes the SDFs themselves will provide the means of joining cloned sequences into a contig.

The patent publication WO95/35505 and U.S. Patents 5,445,943 and 5,410,270 describe scanning multiple alleles of a plurality of loci using hybridization to arrays of oligonucleotides. These techniques are useful for each of the types of mapping discussed above.

Following the procedures described above and using a plurality of the SDFs of the present invention, any individual can be genotyped. These individual genotypes can be used for the identification of particular cultivars, varieties, lines, ecotypes and genetically modified plants or can serve as tools for subsequent genetic studies involving multiple phenotypic traits.

### B.3 Southern Blot Hybridization

The sequences from Table 1 and Table 2, if present can be used as probes for various hybridization techniques. These techniques are useful for detecting target polynucleotides in a sample or for determining whether transgenic plants, seeds or host cells harbor a gene or sequence of interest and thus might be expected to exhibit a particular trait or phenotype.

In addition, the SDFs from the invention can be used to isolate additional members of gene families from the same or different species and/or orthologous genes from the same or different species. This is accomplished by hybridizing an SDF to, for example, a Southern blot containing the appropriate genomic DNA or cDNA. Given the resulting hybridization data, one of ordinary skill in the art could distinguish and isolate the correct DNA fragments by size, restriction sites, sequence and stated hybridization conditions from a gel or from a library.

Identification and isolation of orthologous genes from closely related species and alleles within a species is particularly desirable because of their potential for crop improvement. Many important crop traits, such as the solid content of tomatoes, result from the combined interactions of the products of several genes residing at different loci in the genome. Generally, alleles at each of these loci can make quantitative differences to the

trait. By identifying and isolating numerous alleles for each locus from within or different species, transgenic plants with various combinations of alleles can be created and the effects of the combinations measured. Once a more favorable allele combination has been identified, crop improvement can be accomplished either through biotechnological means or by directed conventional breeding programs (Tanksley et al. *Science* 277:1063(1997)).

5 The results from hybridizations of the SDFs of the invention to, for example, Southern blots containing DNA from another species can also be used to generate restriction fragment maps for the corresponding genomic regions. These maps provide additional 10 information about the relative positions of restriction sites within fragments, further distinguishing mapped DNA from the remainder of the genome.

Physical maps can be made by digesting genomic DNA with different combinations of restriction enzymes.

15 Probes for Southern blotting to distinguish individual restriction fragments can range in size from 15 to 20 nucleotides to several thousand nucleotides. More preferably, the probe is 100 to 1,000 nucleotides long for identifying members of a gene family when it is found that repetitive sequences would complicate the hybridization. For identifying an 20 entire corresponding gene in another species, the probe is more preferably the length of the gene, typically 2,000 to 10,000 nucleotides, but probes 50-1,000 nucleotides long might be used. Some genes, however, might require probes up to 1,500 nucleotides long or overlapping probes constituting the full-length sequence to span their lengths.

25 Also, while it is preferred that the probe be homogeneous with respect to its sequence, it is not necessary. For example, as described below, a probe representing members of a gene family having diverse sequences can be generated using PCR to amplify genomic DNA or RNA templates using primers derived from SDFs that include sequences that define the gene family.

30 For identifying corresponding genes in another species, the next most preferable probe is a cDNA spanning the entire coding sequence, which allows all of the mRNA-coding fragment of the gene to be identified. Probes for Southern blotting can easily be generated from SDFs by making primers having the sequence at the ends of the SDF and using corn or *Arabidopsis* genomic DNA as a template. In instances where the SDF includes sequence conserved among species, primers including the conserved sequence can be used for PCR with genomic DNA from a species of interest to obtain a probe.

Similarly, if the SDF includes a domain of interest, that fragment of the SDF can be used to make primers and, with appropriate template DNA, used to make a probe to identify genes containing the domain. Alternatively, the PCR products can be resolved, for example by gel electrophoresis, and cloned and/or sequenced. Using Southern hybridization, the variants of the domain among members of a gene family, both within and across species, can be examined.

#### B.4.1 Isolating DNA from Related Organisms

The SDFs of the invention can be used to isolate the corresponding DNA from other organisms. Either cDNA or genomic DNA can be isolated. For isolating genomic DNA, a lambda, cosmid, BAC or YAC, or other large insert genomic library from the plant of interest can be constructed using standard molecular biology techniques as described in detail by Sambrook et al. 1989 (Molecular Cloning: A Laboratory Manual, 2<sup>nd</sup> ed. Cold Spring Harbor Laboratory Press, New York) and by Ausubel et al. 1992 (Current Protocols in Molecular Biology, Greene Publishing, New York).

To screen a phage library, for example, recombinant lambda clones are plated out on appropriate bacterial medium using an appropriate *E. coli* host strain. The resulting plaques are lifted from the plates using nylon or nitrocellulose filters. The plaque lifts are processed through denaturation, neutralization, and washing treatments following the standard protocols outlined by Ausubel et al. (1992). The plaque lifts are hybridized to either radioactively labeled or non-radioactively labeled SDF DNA at room temperature for about 16 hours, usually in the presence of 50% formamide and 5X SSC (sodium chloride and sodium citrate) buffer and blocking reagents. The plaque lifts are then washed at 42°C with 1% Sodium Dodecyl Sulfate (SDS) and at a particular concentration of SSC. The SSC concentration used is dependent upon the stringency at which hybridization occurred in the initial Southern blot analysis performed. For example, if a fragment hybridized under medium stringency (e.g., T<sub>m</sub> - 20°C), then this condition is maintained or preferably adjusted to a less stringent condition (e.g., T<sub>m</sub>-30°C) to wash the plaque lifts. Positive clones show detectable hybridization e.g., by exposure to X-ray films or chromogen formation. The positive clones are then subsequently isolated for purification using the same general protocol outlined above. Once the clone is purified, restriction analysis can be conducted to narrow the region corresponding to the gene of interest. The restriction analysis and succeeding subcloning steps can be done using procedures described by, for example Sambrook et al. (1989) cited above.

The procedures outlined for the lambda library are essentially similar to those used for YAC library screening, except that the YAC clones are harbored in bacterial colonies. The YAC clones are plated out at reasonable density on nitrocellulose or nylon filters supported by appropriate bacterial medium in petri plates. Following the growth of the bacterial clones, the filters are processed through the denaturation, neutralization, and washing steps following the procedures of Ausubel et al. 1992. The same hybridization procedures for lambda library screening are followed.

5 To isolate cDNA, similar procedures using appropriately modified vectors are employed. For instance, the library can be constructed in a lambda vector appropriate for cloning cDNA such as  $\lambda$ gt11. Alternatively, the cDNA library can be made in a plasmid vector. cDNA for cloning can be prepared by any of the methods known in the art, but is preferably prepared as described above. Preferably, a cDNA library will include a high proportion of full-length clones.

#### B. 5. Isolating and/or Identifying Orthologous Genes

10 Probes and primers of the invention can be used to identify and/or isolate polynucleotides related to those in Table 1 and Table 2, if present. Related polynucleotides are those that are native to other plant organisms and exhibit either similar sequence or encode polypeptides with similar biological activity. One specific example is an orthologous gene. Orthologous genes have the same functional activity. As such, orthologous genes may be distinguished from homologous genes. The percentage of identity is a function of evolutionary 15 separation and, in closely related species, the percentage of identity can be 98 to 100%. The amino acid sequence of a protein encoded by an orthologous gene can be less than 75% identical, but tends to be at least 75% or at least 80% identical, more preferably at least 90%, most preferably at least 95% identical to the amino acid sequence of the reference protein.

20 To find orthologous genes, the probes are hybridized to nucleic acids from a species of interest under low stringency conditions, preferably one where sequences containing as much as 40-45% mismatches will be able to hybridize. This condition is established by  $T_m - 40^\circ\text{C}$  to  $T_m - 48^\circ\text{C}$  (see below). Blots are then washed under conditions of increasing stringency. It is preferable that the wash stringency be such that sequences that are 85 to 100% identical will hybridize. More preferably, sequences 90 to 100% identical will hybridize and most 25 preferably only sequences greater than 95% identical will hybridize. One of ordinary skill in the art will recognize that, due to degeneracy in the genetic code, amino acid sequences that are identical can be encoded by DNA sequences as little as 67% identical or less. Thus, it is

preferable, for example, to make an overlapping series of shorter probes, on the order of 24 to 45 nucleotides, and individually hybridize them to the same arrayed library to avoid the problem of degeneracy introducing large numbers of mismatches.

As evolutionary divergence increases, genome sequences also tend to diverge.

5 Thus, one of skill will recognize that searches for orthologous genes between more divergent species will require the use of lower stringency conditions compared to searches between closely related species. Also, degeneracy of the genetic code is more of a problem for searches in the genome of a species more distant evolutionarily from the species that is the source of the SDF probe sequences.

10 Therefore the method described in Bouckaert et al., U.S. Ser. No. 60/121,700 Atty. Dkt. No. 2750-117P, Client Dkt. No. 00010.001, filed February 25, 1999, hereby incorporated in its entirety by reference, can be applied to the SDFs of the present invention to isolate related genes from plant species which do not hybridize to the corn *Arabidopsis*, soybean, rice, wheat, and other plant sequences of Table 1 and Table 2, if present.

15 Identification of the relationship of nucleotide or amino acid sequences among plant species can be done by comparing the nucleotide or amino acid sequences of SDFs of the present application with nucleotide or amino acid sequences of other SDFs such as those present in applications listed in the table below:

Country	Attorney No.	Filing Date	Application No.
United States	2750-0301P	9/4/98	60/099,672
United States	2750-0300P	9/4/98	60/099,671
United States	2750-0302P	9/11/98	60/099,933
United States	2750-0304P	9/17/98	60/100,864
United States	2750-0305P	9/18/98	60/101,042
United States	2750-0306P	9/21/98	60/101,255
United States	2750-0307P	9/24/98	60/101,682
United States	2750-0308P	9/30/98	60/102,533
United States	2750-0309P	9/30/98	60/102,460
United States	2750-0311P	10/5/98	60/103,141
United States	2750-0310P	10/5/98	60/103,116
United States	2750-0312P	10/6/98	60/103,215
United States	2750-0313P	10/8/98	60/103,554
United States	2750-0314P	10/9/98	60/103,574
United States	2750-0315P	10/13/98	60/103,907
United States	2750-0316P	10/14/98	60/104,268
United States	2750-0317P	10/16/98	60/104,680
United States	2750-0318P	10/19/98	60/104,828
United States	2750-0319P	10/20/98	60/105,008
United States	2750-0320P	10/21/98	60/105,142
United States	2750-0321P	10/22/98	60/105,533
United States	2750-0322P	10/26/98	60/105,571
United States	2750-0323P	10/27/98	60/105,815

Country	Attorney No.	Filing Date	Application No.
United States	2750-0324P	10/29/98	60/106,105
United States	2750-0325P	10/30/98	60/106,218
United States	2750-0326P	11/2/98	60/106,685
United States	2750-0327P	11/6/98	60/107,282
United States	2750-0329P	11/9/98	60/107,719
United States	2750-0328P	11/9/98	60/107,720
United States	2750-0330P	11/10/98	60/107,836
United States	2750-0331P	11/12/98	60/108,190
United States	2750-0332P	11/16/98	60/108,526
United States	2750-0333P	11/17/98	60/108,901
United States	2750-0334P	11/19/98	60/109,124
United States	2750-0335P	11/19/98	60/109,127
United States	2750-0336P	11/20/98	60/109,267
United States	2750-0337P	11/23/98	60/109,594
United States	2750-0339P	11/25/98	60/110,050
United States	2750-0338P	11/25/98	60/110,053
United States	2750-0340P	11/27/98	60/110,158
United States	2750-0341P	11/30/98	60/110,263
United States	2750-0342P	12/1/98	60/110,495
United States	2750-0343P	12/2/98	60/110,626
United States	2750-0344P	12/3/98	60/110,701
United States	2750-0345P	12/7/98	60/111,339
United States	2750-0346P	12/9/98	60/111,589
United States	2750-0347P	12/10/98	60/111,782
United States	2750-0348P	12/11/98	60/111,812
United States	2750-0349P	12/14/98	60/112,096
United States	2750-0350P	12/15/98	60/112,224
United States	2750-0351P	12/16/98	60/112,624
United States	2750-0352P	12/17/98	60/112,862
United States	2750-0353P	12/18/98	60/112,912
United States	2750-0354P	12/21/98	60/113,248
United States	2750-0355P	12/22/98	60/113,522
United States	2750-0356P	12/23/98	60/113,826
United States	2750-0357P	12/28/98	60/113,998
United States	2750-0358P	12/29/98	60/114,384
United States	2750-0359P	12/30/98	60/114,455
United States	2750-0360P	1/4/99	60/114,740
United States	2750-0361P	1/6/99	60/114,866
United States	2750-0367P	1/7/99	60/115,154
United States	2750-0364P	1/7/99	60/115,151
United States	2750-0363P	1/7/99	60/115,152
United States	2750-0362P	1/7/99	60/115,153
United States	2750-0366P	1/7/99	60/115,156
United States	2750-0365P	1/7/99	60/115,155
United States	2750-0370P	1/8/99	60/115,293
United States	2750-0369P	1/8/99	60/115,365
United States	2750-0368P	1/8/99	60/115,364
United States	2750-0371P	1/11/99	60/115,339
United States	2750-0372P	1/12/99	60/115,518
United States	2750-0373P	1/13/99	60/115,847

Country	Attorney No.	Filing Date	Application No.
United States	2750-0374P	1/14/99	60/115,905
United States	2750-0376P	1/15/99	60/116,384
United States	2750-0375P	1/15/99	60/116,383
United States	2750-0377P	1/19/99	60/116,329
United States	2750-0378P	1/19/99	60/116,340
United States	2750-0379P	1/21/99	60/116,674
United States	2750-0380P	1/21/99	60/116,672
United States	2750-0382P	1/22/99	60/116,962
United States	2750-0381P	1/22/99	60/116,960
United States	2750-0383P	1/28/99	60/117,756
United States	2750-0384P	2/3/99	60/118,672
United States	2750-0385P	2/4/99	60/118,808
United States	2750-0386P	2/5/99	60/118,778
United States	2750-0387P	2/8/99	60/119,029
United States	2750-0388P	2/9/99	60/119,332
United States	2750-0389P	2/10/99	60/119,462
United States	2750-0391P	2/12/99	60/119,922
United States	2750-0393P	2/16/99	60/120,198
United States	2750-0392P	2/16/99	60/120,196
United States	2750-0394P	2/18/99	60/120,583
United States	2750-0395P	2/22/99	60/121,072
United States	2750-0396P	2/23/99	60/121,334
United States	2750-0397P	2/24/99	60/121,470
United States	2750-0390P	2/25/99	60/121,825
United States	2750-0398P	2/25/99	60/121,704
United States	2750-0399P	2/26/99	60/122,107
United States	2750-0400P	3/1/99	60/122,266
United States	2750-0401P	3/2/99	60/122,568
United States	2750-0402P	3/3/99	60/122,611
United States	2750-0403P	3/4/99	60/121,775
United States	2750-0405P	3/5/99	60/123,180
United States	2750-0404P	3/5/99	60/123,534
United States	2750-0406P	3/9/99	60/123,680
United States	2750-0407P	3/9/99	60/123,548
United States	2750-0409P	3/10/99	60/123,726
United States	2750-0408P	3/10/99	60/123,715
United States	2750-0410P	3/11/99	60/124,263
United States	2750-0411P	3/12/99	60/123,941
United States	2750-0412P	3/23/99	60/125,788
United States	2750-0413P	3/25/99	60/126,264
United States	2750-0414P	3/29/99	60/126,785
United States	2750-0415P	4/1/99	60/127,462
United States	2750-0416P	4/6/99	60/128,234
United States	2750-0417P	4/8/99	60/128,714
United States	2750-0418P	4/16/99	60/129,845
United States	2750-0420P	4/19/99	60/130,077
United States	2750-0421P	4/21/99	60/130,449
United States	2750-0303P	4/23/99	60/130,510
United States	2750-0422P	4/23/99	60/130,891
United States	2750-0423P	4/28/99	60/131,449

Country	Attorney No.	Filing Date	Application No.
United States	2750-0424P	4/30/99	60/132,407
United States	2750-0425P	4/30/99	60/132,048
United States	2750-0426P	5/4/99	60/132,484
United States	2750-0427P	5/5/99	60/132,485
United States	2750-0428P	5/6/99	60/132,487
United States	2750-0429P	5/6/99	60/132,486
United States	2750-0430P	5/7/99	60/132,863
United States	2750-0431P	5/11/99	60/134,256
United States	2750-0433P	5/14/99	60/134,221
United States	2750-0432P	5/14/99	60/134,370
United States	2750-0434P	5/14/99	60/134,219
United States	2750-0435P	5/14/99	60/134,218
United States	2750-0436P	5/18/99	60/134,768
United States	2750-0437P	5/19/99	60/134,941
United States	2750-0438P	5/20/99	60/135,124
United States	2750-0439P	5/21/99	60/135,353
United States	2750-0440P	5/24/99	60/135,629
United States	2750-0441P	5/25/99	60/136,021
United States	2750-0442P	5/27/99	60/136,392
United States	2750-0444P	5/28/99	60/136,782
United States	2750-0445P	6/1/99	60/137,222
United States	2750-0446P	6/3/99	60/137,528
United States	2750-0447P	6/4/99	60/137,502
United States	2750-0449P	6/7/99	60/137,724
United States	2750-0450P	6/8/99	60/138,094
United States	2750-0458P	6/10/99	60/138,847
United States	2750-0457P	6/10/99	60/138,540
United States	2750-0463P	6/14/99	60/139,119
United States	2750-0462P	6/16/99	60/139,452
United States	2750-0461P	6/16/99	60/139,453
United States	2750-0464P	6/17/99	60/139,492
United States	2750-0454P	6/18/99	60/139,457
United States	2750-0460P	6/18/99	60/139,455
United States	2750-0466P	6/18/99	60/139,750
United States	2750-0465P	6/18/99	60/139,763
United States	2750-0459P	6/18/99	60/139,463
United States	2750-0455P	6/18/99	60/139,460
United States	2750-0453P	6/18/99	60/139,462
United States	2750-0452P	6/18/99	60/139,461
United States	2750-0451P	6/18/99	60/139,459
United States	2750-0448P	6/18/99	60/139,454
United States	2750-0443P	6/18/99	60/139,458
United States	2750-0456P	6/18/99	60/139,456
United States	2750-0467P	6/21/99	60/139,817
United States	2750-0468P	6/22/99	60/139,899
United States	2750-0469P	6/23/99	60/140,354
United States	2750-0470P	6/23/99	60/140,353
United States	2750-0471P	6/24/99	60/140,695
United States	2750-0472P	6/28/99	60/140,823
United States	2750-0473P	6/29/99	60/140,991

Country	Attorney No.	Filing Date	Application No.
United States	2750-0474P	6/30/99	60/141,287
United States	2750-0475P	7/1/99	60/141,842
United States	2750-0476P	7/1/99	60/142,154
United States	2750-0477P	7/2/99	60/142,055
United States	2750-0478P	7/6/99	60/142,390
United States	2750-0479P	7/8/99	60/142,803
United States	2750-0480P	7/9/99	60/142,920
United States	2750-0481P	7/12/99	60/142,977
United States	2750-0482P	7/13/99	60/143,542
United States	2750-0489P	7/14/99	60/143,624
United States	2750-0490P	7/15/99	60/144,005
United States	2750-0486P	7/16/99	60/144,085
United States	2750-0485P	7/16/99	60/144,086
United States	2750-0494P	7/19/99	60/144,333
United States	2750-0497P	7/19/99	60/144,325
United States	2750-0488P	7/19/99	60/144,332
United States	2750-0495P	7/19/99	60/144,335
United States	2750-0492P	7/19/99	60/144,331
United States	2750-0496P	7/19/99	60/144,334
United States	2750-0499P	7/20/99	60/144,352
United States	2750-0500P	7/20/99	60/144,632
United States	2750-0502P	7/20/99	60/144,884
United States	2750-0483P	7/21/99	60/145,088
United States	2750-0484P	7/21/99	60/145,086
United States	2750-0503P	7/21/99	60/144,814
United States	2750-0491P	7/22/99	60/145,085
United States	2750-0493P	7/22/99	60/145,087
United States	2750-0487P	7/22/99	60/145,089
United States	2750-0504P	7/22/99	60/145,192
United States	2750-0505P	7/23/99	60/145,218
United States	2750-0498P	7/23/99	60/145,145
United States	2750-0501P	7/23/99	60/145,224
United States	2750-0506P	7/26/99	60/145,276
United States	2750-0508P	7/27/99	60/145,919
United States	2750-0509P	7/27/99	60/145,913
United States	2750-0507P	7/27/99	60/145,918
United States	2750-0510P	7/28/99	60/145,951
United States	2750-0511P	8/2/99	60/146,388
United States	2750-0512P	8/2/99	60/146,389
United States	2750-0513P	8/2/99	60/146,386
United States	2750-0514P	8/3/99	60/147,038
United States	2750-0517P	8/4/99	60/147,302
United States	2750-0515P	8/4/99	60/147,204
United States	2750-0518P	8/5/99	60/147,260
United States	2750-0519P	8/5/99	60/147,192
United States	2750-0516P	8/6/99	60/147,303
United States	2750-0520P	8/6/99	60/147,416
United States	2750-0523P	8/9/99	60/147,935
United States	2750-0521P	8/9/99	60/147,493
United States	2750-0522P	8/10/99	60/148,171

Country	Attorney No.	Filing Date	Application No.
United States	2750-0524P	8/11/99	60/148,319
United States	2750-0526P	8/12/99	60/148,342
United States	2750-0527P	8/12/99	60/148,340
United States	2750-0528P	8/12/99	60/148,337
United States	2750-0530P	8/12/99	60/148,341
United States	2750-0525P	8/12/99	60/148,347
United States	2750-0529P	8/13/99	60/148,565
United States	2750-0532P	8/13/99	60/148,684
United States	2750-0531P	8/16/99	60/149,368
United States	2750-0534P	8/17/99	60/149,928
United States	2750-0537P	8/17/99	60/149,175
United States	2750-0536P	8/17/99	60/149,925
United States	2750-0535P	8/17/99	60/149,926
United States	2750-0533P	8/17/99	60/149,927
United States	2750-0538P	8/18/99	60/149,426
United States	2750-0539P	8/20/99	60/149,722
United States	2750-0541P	8/20/99	60/149,929
United States	2750-0542P	8/20/99	60/149,723
United States	2750-0540P	8/23/99	60/149,930
United States	2750-0543P	8/23/99	60/149,902
United States	2750-0544P	8/25/99	60/150,566
United States	2750-0547P	8/26/99	60/150,884
United States	2750-0548P	8/27/99	60/151,080
United States	2750-0546P	8/27/99	60/151,066
United States	2750-0545P	8/27/99	60/151,065
United States	2750-0549P	8/30/99	60/151,303
United States	2750-0552P	8/31/99	60/151,438
United States	2750-0553P	9/1/99	60/151,930
United States	2750-0550P	9/3/99	60/151,631
United States	2750-0554P	9/7/99	60/152,363
United States	2750-0555P	9/10/99	60/153,070
United States	2750-0556P	9/13/99	60/153,758
United States	2750-0557P	9/15/99	60/154,018
United States	2750-0558P	9/16/99	60/154,039
United States	2750-0559P	9/20/99	60/154,779
United States	2750-0560P	9/22/99	60/155,139
United States	2750-0561P	9/23/99	60/155,486
United States	2750-0562P	9/24/99	60/155,659
United States	2750-0563P	9/28/99	60/156,458
United States	2750-0564P	9/29/99	60/156,596
United States	2750-0570P	10/4/99	60/157,117
United States	2750-0565P	10/5/99	60/157,198
United States	2750-0566P	10/5/99	60/157,922
United States	2750-0571P	10/5/99	60/157,753
United States	2750-0572P	10/6/99	60/157,865
United States	2750-0575P	10/7/99	60/158,029
United States	2750-0576P	10/8/99	60/158,232
United States	2750-0577P	10/12/99	60/158,369
United States	2750-0574P	10/13/99	60/159,295
United States	2750-0583P	10/13/99	60/159,294

Country	Attorney No.	Filing Date	Application No.
United States	2750-0579P	10/13/99	60/159,293
United States	2750-0578P	10/14/99	60/159,331
United States	2750-0573P	10/14/99	60/159,330
United States	2750-0580P	10/14/99	60/159,638
United States	2750-0581P	10/14/99	60/159,637
United States	2750-0582P	10/14/99	60/159,329
United States	2750-0584P	10/18/99	60/159,584
United States	2750-0586P	10/21/99	60/160,814
United States	2750-0590P	10/21/99	60/160,767
United States	2750-0589P	10/21/99	60/160,768
United States	2750-0587P	10/21/99	60/160,770
United States	2750-0585P	10/21/99	60/160,815
United States	2750-0588P	10/21/99	60/160,741
United States	2750-0591P	10/22/99	60/160,980
United States	2750-0592P	10/22/99	60/160,989
United States	2750-0593P	10/22/99	60/160,981
United States	2750-0594P	10/25/99	60/161,405
United States	2750-0596P	10/25/99	60/161,404
United States	2750-0595P	10/25/99	60/161,406
United States	2750-0597P	10/26/99	60/161,361
United States	2750-0598P	10/26/99	60/161,360
United States	2750-0599P	10/26/99	60/161,359
United States	2750-0602P	10/28/99	60/161,992
United States	2750-0601P	10/28/99	60/161,920
United States	2750-0600P	10/28/99	09/428,944
United States	2750-0603P	10/28/99	60/161,993
United States	2750-0604P	10/29/99	60/162,143
United States	2750-0606P	10/29/99	60/162,228
United States	2750-0605P	10/29/99	60/162,142
United States	2750-0608P	11/1/99	60/162,891
United States	2750-0607P	11/1/99	60/162,894
United States	2750-0609P	11/1/99	60/162,895
United States	2750-0610P	11/2/99	60/163,093
United States	2750-0611P	11/2/99	60/163,092
United States	2750-0612P	11/2/99	60/163,091
United States	2750-0613P	11/3/99	60/163,249
United States	2750-0615P	11/3/99	60/163,281
United States	2750-0614P	11/3/99	60/163,248
United States	2750-0616P	11/4/99	60/163,379
United States	2750-0617P	11/4/99	60/163,381
United States	2750-0618P	11/4/99	60/163,380
United States	2750-0619P	11/8/99	60/164,146
United States	2750-0620P	11/8/99	60/164,151
United States	2750-0621P	11/8/99	60/164,150
United States	2750-0625P	11/9/99	60/164,259
United States	2750-0623P	11/9/99	60/164,260
United States	2750-0630P	11/10/99	60/164,548
United States	2750-0628P	11/10/99	60/164,544
United States	2750-0629P	11/10/99	60/164,545
United States	2750-0626P	11/10/99	60/164,321

Country	Attorney No.	Filing Date	Application No.
United States	2750-0627P	11/10/99	60/164,318
United States	2750-0622P	11/10/99	60/164,319
United States	2750-0624P	11/10/99	60/164,317
United States	2750-0634P	11/12/99	60/164,870
United States	2750-0635P	11/12/99	60/164,959
United States	2750-0633P	11/12/99	60/164,960
United States	2750-0632P	11/12/99	60/164,871
United States	2750-0636P	11/12/99	60/164,962
United States	2750-0631P	11/12/99	60/164,961
United States	2750-0638P	11/15/99	60/164,929
United States	2750-0639P	11/15/99	60/164,926
United States	2750-0637P	11/15/99	60/164,927
United States	2750-0641P	11/16/99	60/165,671
United States	2750-0640P	11/16/99	60/165,669
United States	2750-0642P	11/16/99	60/165,661
United States	2750-0643P	11/17/99	60/165,919
United States	2750-0644P	11/17/99	60/165,918
United States	2750-0645P	11/17/99	60/165,911
United States	2750-0648P	11/18/99	60/166,158
United States	2750-0646P	11/18/99	60/166,157
United States	2750-0647P	11/18/99	60/166,173
United States	2750-0651P	11/19/99	60/166,412
United States	2750-0650P	11/19/99	60/166,411
United States	2750-0649P	11/19/99	60/166,419
United States	2750-0652P	11/22/99	60/166,733
United States	2750-0653P	11/22/99	60/166,750
United States	2750-0655P	11/23/99	60/167,362
United States	2750-0654P	11/24/99	60/167,382
United States	2750-0656P	11/24/99	60/167,233
United States	2750-0657P	11/24/99	60/167,234
United States	2750-0658P	11/24/99	60/167,235
United States	2750-0659P	11/30/99	60/167,904
United States	2750-0660P	11/30/99	60/167,908
United States	2750-0661P	11/30/99	60/167,902
United States	2750-0663P	12/1/99	60/168,232
United States	2750-0665P	12/1/99	60/168,231
United States	2750-0662P	12/1/99	09/451,320
United States	2750-0664P	12/1/99	60/168,233
United States	2750-0668P	12/2/99	60/168,548
United States	2750-0667P	12/2/99	60/168,549
United States	2750-0666P	12/2/99	60/168,546
United States	2750-0669P	12/3/99	60/168,675
United States	2750-0670P	12/3/99	60/168,673
United States	2750-0671P	12/3/99	60/168,674
United States	2750-0672P	12/7/99	60/169,298
United States	2750-0674P	12/7/99	60/169,302
United States	2750-0673P	12/7/99	60/169,278
United States	2750-0675P	12/8/99	60/169,692
United States	2750-0676P	12/8/99	60/169,691
United States	2750-0677P	12/16/99	60/171,107

Country	Attorney No.	Filing Date	Application No.
United States	2750-0678P	12/16/99	60/171,114
United States	2750-0679P	12/16/99	60/171,098
United States	2750-0683P	1/4/00	09/478,081
International	2750-0686F(PC)	1/7/00	00/004,66
United States	2750-0684P	1/7/00	09/479,221
United States	2750-0681P	1/19/00	60/176,866
United States	2750-0685P	1/19/00	60/176,867
United States	2750-0688P	1/20/00	60/176,910
United States	2750-0689P	1/26/00	60/178,166
United States	2750-0690P	1/27/00	60/178,547
United States	2750-0682P	1/27/00	60/178,546
United States	2750-0680P	1/27/00	60/178,544
United States	2750-0687P	1/27/00	60/178,545
United States	2750-0691P	1/27/00	60/177,666
United States	2750-0692P	1/28/00	60/178,754
United States	2750-0693P	1/28/00	60/178,755
United States	2750-0695P	2/1/00	60/179,395
United States	2750-0696P	2/1/00	60/179,388
United States	2750-0697P	2/3/00	60/180,039
United States	2750-0698P	2/3/00	60/180,139
United States	2750-0694P	2/3/00	09/497,191
United States	2750-0700P	2/4/00	60/180,207
United States	2750-0699P	2/4/00	60/180,206
United States	2750-0701P	2/7/00	60/180,695
United States	2750-0702P	2/7/00	60/180,696
United States	2750-0703P	2/9/00	60/181,228
United States	2750-0704P	2/9/00	60/181,214
United States	2750-0706P	2/10/00	60/181,551
United States	2750-0705P	2/10/00	60/181,476
United States	2750-0713P	2/15/00	60/182,478
United States	2750-0712P	2/15/00	60/182,512
United States	2750-0708P	2/15/00	60/182,516
United States	2750-0707P	2/15/00	60/182,477
United States	2750-0714P	2/17/00	60/183,166
United States	2750-0715P	2/17/00	60/183,165
United States	2750-0716P	2/24/00	60/184,667
United States	2750-0717P	2/24/00	60/184,658
United States	2750-0718P	2/25/00	60/185,140
Canada	2750-0709F(CA)	2/25/00	23/006,92
Europe	2750-0709F(EP)	2/25/00	00/301,439
United States	2750-0720P	2/25/00	60/185,119
United States	2750-0719P	2/25/00	60/185,118
United States	2750-0709P	2/25/00	09/513,996
Mexico	2750-0709F(MX)	2/25/00	00/001,973
United States	2750-0722P	2/28/00	60/185,396
United States	2750-0723P	2/28/00	60/185,397
United States	2750-0721P	2/28/00	60/185,398
United States	2750-0724P	2/29/00	60/185,750
United States	2750-0725P	3/1/00	60/186,283
United States	2750-0727P	3/1/00	60/186,277

Country	Attorney No.	Filing Date	Application No.
United States	2750-0710P	3/1/00	09/517,537
United States	2750-0726P	3/1/00	60/186,296
United States	2750-0728P	3/2/00	60/187,178
United States	2750-0729P	3/2/00	60/186,386
United States	2750-0730P	3/2/00	60/186,387
United States	2750-0711P	3/2/00	60/186,390
United States	2750-0731P	3/3/00	60/186,670
United States	2750-0732P	3/3/00	60/186,748
United States	2750-0733P	3/3/00	60/186,669
United States	2750-0734P	3/7/00	60/187,378
United States	2750-0735P	3/7/00	60/187,379
United States	2750-0737P	3/8/00	60/187,888
United States	2750-0736P	3/8/00	60/187,896
United States	2750-0738P	3/9/00	60/187,985
United States	2750-0741P	3/10/00	60/188,174
United States	2750-0742P	3/10/00	60/188,185
United States	2750-0740P	3/10/00	60/188,186
United States	2750-0739P	3/10/00	60/188,187
United States	2750-0743P	3/10/00	60/188,175
United States	2750-0744P	3/13/00	60/188,687
United States	2750-0745P	3/14/00	60/189,080
United States	2750-0746P	3/14/00	60/189,052
United States	2750-0747P	3/15/00	60/189,460
United States	2750-0748P	3/15/00	60/189,461
United States	2750-0749P	3/15/00	60/189,462
United States	2750-0755P	3/16/00	60/189,953
United States	2750-0752P	3/16/00	60/189,948
United States	2750-0756P	3/16/00	60/189,959
United States	2750-0751P	3/16/00	60/189,947
United States	2750-0753P	3/16/00	60/190,121
United States	2750-0754P	3/16/00	60/189,958
United States	2750-0750P	3/16/00	60/190,120
United States	2750-0757P	3/17/00	60/189,965
United States	2750-0762P	3/20/00	60/190,089
United States	2750-0759P	3/20/00	60/190,070
United States	2750-0758P	3/20/00	60/190,069
United States	2750-0761P	3/20/00	60/190,545
United States	2750-0760P	3/20/00	60/190,090
United States	2750-0763P	3/22/00	60/191,084
United States	2750-0764P	3/22/00	60/191,097
United States	2750-0767P	3/23/00	60/191,545
United States	2750-0766P	3/23/00	60/191,543
United States	2750-0765P	3/23/00	60/191,549
United States	2750-0768P	3/24/00	60/191,826
United States	2750-0769P	3/24/00	60/191,823
United States	2750-0770P	3/24/00	60/191,825
United States	2750-0771P	3/27/00	60/192,420
United States	2750-0772P	3/27/00	60/192,421
United States	2750-0773P	3/27/00	60/192,308
United States	2750-0775P	3/29/00	60/192,940

Country	Attorney No.	Filing Date	Application No.
United States	2750-0776P	3/29/00	60/192,941
United States	2750-0774P	3/29/00	60/192,855
United States	2750-0778P	3/30/00	60/193,244
United States	2750-0779P	3/30/00	60/193,245
United States	2750-0777P	3/30/00	60/193,243
United States	2750-0780P	3/31/00	60/193,469
United States	2750-0781P	3/31/00	60/193,453
United States	2750-0782P	3/31/00	60/193,455
United States	2750-0785P	4/4/00	60/194,385
United States	2750-0787P	4/4/00	60/194,398
United States	2750-0786P	4/4/00	60/194,404
United States	2750-0790P	4/5/00	60/194,683
United States	2750-0792P	4/5/00	60/194,698
United States	2750-0791P	4/5/00	60/194,697
United States	2750-0789P	4/5/00	60/194,682
United States	2750-0796P	4/6/00	60/195,045
United States	2750-0783P	4/6/00	09/543,680
United States	2750-0795P	4/6/00	60/194,885
United States	2750-0794P	4/6/00	60/194,872
United States	2750-0784P	4/6/00	60/194,884
United States	2750-0793P	4/6/00	60/194,874
United States	2750-0799P	4/7/00	60/195,257
United States	2750-0797P	4/7/00	60/195,258
United States	2750-0798P	4/7/00	60/195,283
United States	2750-0804P	4/11/00	60/196,089
United States	2750-0803P	4/11/00	60/196,169
United States	2750-0802P	4/11/00	60/196,168
United States	2750-0801P	4/11/00	60/196,211
United States	2750-0809P	4/12/00	60/196,486
United States	2750-0800P	4/12/00	60/196,212
United States	2750-0805P	4/12/00	60/196,483
United States	2750-0806P	4/12/00	60/196,487
United States	2750-0807P	4/12/00	60/196,289
United States	2750-0808P	4/12/00	60/196,485
United States	2750-0811P	4/13/00	60/196,213
United States	2750-0810P	4/14/00	60/197,869
United States	2750-0814P	4/14/00	60/197,397
United States	2750-0819P	4/17/00	60/197,671
United States	2750-0817P	4/17/00	60/198,268
United States	2750-0812P	4/17/00	60/197,870
United States	2750-0813P	4/17/00	60/197,871
United States	2750-0818P	4/17/00	60/198,133
United States	2750-0816P	4/17/00	60/197,678
United States	2750-0815P	4/17/00	60/197,687
United States	2750-0820P	4/19/00	60/198,400
United States	2750-0821P	4/19/00	60/198,386
United States	2750-0822P	4/19/00	60/198,373
United States	2750-0823P	4/20/00	60/198,629
United States	2750-0824P	4/20/00	60/198,619
United States	2750-0825P	4/20/00	60/198,623

Country	Attorney No.	Filing Date	Application No.
United States	2750-0826P	4/21/00	60/198,765
United States	2750-0828P	4/21/00	60/198,763
United States	2750-0827P	4/21/00	60/198,767
United States	2750-0831P	4/24/00	60/199,122
United States	2750-0829P	4/24/00	60/199,123
United States	2750-0830P	4/24/00	60/199,124
United States	2750-0833P	4/26/00	60/200,031
United States	2750-0832P	4/26/00	60/200,034
United States	2750-0835P	4/26/00	60/199,818
United States	2750-0834P	4/26/00	60/199,828
United States	2750-0837P	4/27/00	60/200,102
United States	2750-0836P	4/27/00	60/200,103
United States	2750-0844P	4/28/00	60/200,373
United States	2750-0846P	4/28/00	60/200,773
United States	2750-0788P	4/28/00	09/559,232
United States	2750-0839P	5/1/00	60/200,879
United States	2750-0848P	5/1/00	60/200,761
United States	2750-0847P	5/1/00	60/200,762
United States	2750-0845P	5/1/00	60/201,016
United States	2750-0843P	5/1/00	60/200,763
United States	2750-0840P	5/1/00	60/200,885
United States	2750-0841P	5/1/00	60/201,017
United States	2750-0842P	5/1/00	60/201,018
United States	2750-0850P	5/2/00	60/201,305
United States	2750-0838P	5/2/00	60/201,275
United States	2750-0849P	5/2/00	60/201,279
United States	2750-0856P	5/4/00	60/201,751
United States	2750-0852P	5/4/00	09/566,262
United States	2750-0857P	5/4/00	60/201,740
United States	2750-0858P	5/4/00	60/201,750
United States	2750-0861P	5/5/00	60/202,180
United States	2750-0860P	5/5/00	60/202,112
United States	2750-0859P	5/5/00	60/202,178
United States	2750-0855P	5/5/00	09/565,310
United States	2750-0854P	5/5/00	09/565,307
United States	2750-0853P	5/5/00	09/565,309
United States	2750-0851P	5/5/00	09/565,308
United States	2750-0863P	5/9/00	60/202,636
United States	2750-0864P	5/9/00	60/202,915
United States	2750-0865P	5/9/00	60/202,919
United States	2750-0866P	5/9/00	60/202,634
United States	2750-0862P	5/9/00	60/202,914
United States	2750-0878P	5/10/00	60/202,968
United States	2750-0877P	5/10/00	60/202,969
United States	2750-0879P	5/10/00	60/202,963
United States	2750-0868P	5/11/00	60/203,672
United States	2750-0871P	5/11/00	09/572,408
United States	2750-0870P	5/11/00	60/203,622
United States	2750-0867P	5/11/00	60/203,671
United States	2750-0880P	5/11/00	60/203,458

Country	Attorney No.	Filing Date	Application No.
United States	2750-0881P	5/11/00	60/203,457
United States	2750-0882P	5/11/00	60/203,279
United States	2750-0869P	5/11/00	60/203,669
United States	2750-0875P	5/12/00	09/570,581
United States	2750-0883P	5/12/00	60/203,911
United States	2750-0872P	5/12/00	09/570,768
United States	2750-0873P	5/12/00	09/570,582
United States	2750-0874P	5/12/00	09/570,738
United States	2750-0884P	5/12/00	60/203,916
United States	2750-0885P	5/12/00	60/203,915
United States	2750-0886P	5/15/00	60/204,395
United States	2750-0888P	5/15/00	60/204,122
United States	2750-0887P	5/15/00	60/204,388
United States	2750-0891P	5/16/00	60/204,568
United States	2750-0892P	5/16/00	60/204,569
United States	2750-0893P	5/17/00	60/204,830
United States	2750-0894P	5/17/00	60/204,829
United States	2750-0890P	5/17/00	60/205,325
United States	2750-0889P	5/17/00	60/205,233
United States	2750-0896P	5/18/00	60/205,058
United States	2750-0895P	5/18/00	60/205,201
United States	2750-0876P	5/18/00	09/573,655
United States	2750-0897P	5/19/00	60/205,242
United States	2750-0898P	5/19/00	60/205,243
United States	2750-0899P	5/22/00	60/205,574
United States	2750-0900P	5/22/00	60/205,572
United States	2750-0901P	5/22/00	60/205,576
United States	2750-0903P	5/23/00	60/206,319
United States	2750-0902P	5/23/00	60/206,316
United States	2750-0905P	5/24/00	60/206,545
United States	2750-0904P	5/24/00	60/206,553
United States	2750-0906P	5/25/00	60/206,988
United States	2750-0907P	5/26/00	60/207,367
United States	2750-0908P	5/26/00	60/207,243
United States	2750-0910P	5/26/00	60/207,239
United States	2750-0911P	5/26/00	60/207,354
United States	2750-0909P	5/26/00	60/207,242
United States	2750-0912P	5/30/00	60/207,291
United States	2750-0913P	5/30/00	60/207,452
United States	2750-0914P	5/30/00	60/207,329
United States	2750-0918P	6/1/00	60/208,648
United States	2750-0921P	6/1/00	60/208,312
United States	2750-0919P	6/1/00	60/208,324
United States	2750-0916P	6/1/00	60/208,421
United States	2750-0920P	6/1/00	60/208,329
United States	2750-0917P	6/2/00	60/208,649
United States	2750-0915P	6/2/00	60/209,338
United States	2750-0922P	6/5/00	60/208,919
United States	2750-0923P	6/5/00	60/208,910
United States	2750-0924P	6/5/00	60/208,918

Country	Attorney No.	Filing Date	Application No.
United States	2750-0925P	6/5/00	60/208,917
United States	2750-0926P	6/5/00	60/208,921
United States	2750-0927P	6/5/00	60/208,920
United States	2750-0929P	6/8/00	60/210,008
United States	2750-0930P	6/8/00	60/210,012
United States	2750-0931P	6/8/00	60/210,006
United States	2750-0928P	6/9/00	09/592,459
United States	2750-0933P	6/9/00	60/210,564
United States	2750-0932P	6/9/00	60/210,670
United States	2750-0936P	6/13/00	60/211,214
United States	2750-0937P	6/13/00	60/211,210
United States	2750-0935P	6/13/00	60/211,213
United States	2750-0934P	6/14/00	09/593,710
United States	2750-0940P	6/15/00	60/211,538
United States	2750-0938P	6/15/00	60/211,539
United States	2750-0939P	6/15/00	60/211,540
United States	2750-0948P	6/16/00	09/595,329
United States	2750-0952P	6/16/00	09/594,597
United States	2750-0953P	6/16/00	09/595,298
United States	2750-0955P	6/16/00	09/595,331
United States	2750-0954P	6/16/00	09/594,599
United States	2750-0942P	6/16/00	09/595,326
United States	2750-0944P	6/16/00	09/595,330
United States	2750-0943P	6/16/00	09/596,577
United States	2750-0947P	6/16/00	09/595,335
United States	2750-0945P	6/16/00	09/595,333
United States	2750-0946P	6/16/00	09/595,328
United States	2750-0951P	6/16/00	09/594,595
United States	2750-0941P	6/16/00	09/595,334
United States	2750-0950P	6/16/00	09/594,598
United States	2750-0949P	6/16/00	09/595,332
United States	2750-0957P	6/19/00	60/212,649
United States	2750-0958P	6/19/00	60/212,623
United States	2750-0956P	6/19/00	60/212,414
United States	2750-0959P	6/20/00	60/212,677
United States	2750-0960P	6/20/00	60/212,713
United States	2750-0961P	6/20/00	60/212,727
United States	2750-0971P	6/21/00	09/602,660
United States	2750-0962P	6/22/00	60/213,271
United States	2750-0967P	6/22/00	60/213,270
United States	2750-0966P	6/22/00	60/213,220
United States	2750-0972P	6/22/00	09/602,152
United States	2750-0965P	6/22/00	60/213,221
United States	2750-0963P	6/22/00	60/213,249
United States	2750-0964P	6/22/00	60/213,195
United States	2750-0973P	6/23/00	09/602,025
United States	2750-0975P	6/23/00	09/602,016
United States	2750-1036P	6/27/00	60/214,535
United States	2750-0968P	6/27/00	60/214,760
United States	2750-0969P	6/27/00	60/214,762

Country	Attorney No.	Filing Date	Application No.
United States	2750-0970P	6/27/00	60/214,524
United States	2750-1035P	6/27/00	60/214,534
United States	2750-1038P	6/28/00	60/214,800
United States	2750-0976P	6/28/00	09/605,843
United States	2750-1039P	6/28/00	60/214,799
United States	2750-0977P	6/29/00	09/606,181
United States	2750-0981P	6/30/00	09/609,198
United States	2750-0979P	6/30/00	09/607,081
United States	2750-0980P	6/30/00	09/610,157
United States	2750-1040P	6/30/00	60/215,775
United States	2750-0978P	6/30/00	09/608,960
United States	2750-1041P	6/30/00	60/215,127
United States	2750-1043P	7/5/00	60/216,362
United States	2750-1042P	7/5/00	60/216,361
United States	2750-0982P	7/6/00	09/611,409
United States	2750-0983P	7/7/00	09/612,645
United States	2750-0984P	7/7/00	09/613,547
United States	2750-1044P	7/11/00	60/217,385
United States	2750-1046P	7/11/00	60/217,384
United States	2750-1045P	7/11/00	60/217,476
United States	2750-0985P	7/12/00	09/615,007
United States	2750-1050P	7/12/00	60/217,754
United States	2750-1052P	7/12/00	06/218,548
United States	2750-0986P	7/13/00	09/615,748
United States	2750-1054P	7/13/00	60/217,846
United States	2750-1048P	7/14/00	60/218,566
United States	2750-0988P	7/14/00	09/617,203
United States	2750-1061P	7/14/00	09/614,450
United States	2750-1060P	7/14/00	09/614,388
United States	2750-0987P	7/14/00	09/617,525
United States	2750-1057P	7/18/00	60/219,033
United States	2750-1055P	7/18/00	60/219,021
United States	2750-1056P	7/18/00	60/219,004
United States	2750-0989P	7/19/00	09/620,421
United States	2750-1062P	7/19/00	09/617,683
United States	2750-1063P	7/19/00	09/617,682
United States	2750-1064P	7/19/00	09/617,681
United States	2750-1066P	7/20/00	09/620,978
United States	2750-1065P	7/20/00	09/620,998
United States	2750-0990P	7/20/00	09/621,323
United States	2750-1069P	7/21/00	09/620,313
United States	2750-0992P	7/21/00	09/621,660
United States	2750-1072P	7/21/00	09/621,900
United States	2750-1073P	7/21/00	09/620,314
United States	2750-1068P	7/21/00	09/620,393
United States	2750-1071P	7/21/00	09/620,390
United States	2750-0993P	7/21/00	09/621,902
United States	2750-1070P	7/21/00	09/620,111
United States	2750-0991P	7/21/00	09/621,630
United States	2750-1067P	7/21/00	09/620,394

Country	Attorney No.	Filing Date	Application No.
United States	2750-1058P	7/25/00	60/220,814
United States	2750-1059P	7/25/00	60/220,467
United States	2750-1079P	7/25/00	60/220,811
United States	2750-1080P	7/25/00	60/220,484
United States	2750-1081P	7/25/00	60/220,652
United States	2750-0994P	7/26/00	09/616,628
United States	2750-0995P	7/27/00	09/628,986
United States	2750-1075P	7/27/00	09/628,987
United States	2750-1074P	7/27/00	09/628,984
United States	2750-0996P	7/28/00	09/628,552
United States	2750-1256P	8/1/00	60/223,114
United States	2750-1049P	8/1/00	60/223,115
United States	2750-1077P	8/2/00	09/630,442
United States	2750-0997P	8/2/00	09/632,340
United States	2750-1076P	8/2/00	09/628,985
United States	2750-1051P	8/3/00	60/223,101
United States	2750-0998P	8/3/00	09/632,349
United States	2750-1258P	8/3/00	60/223,099
United States	2750-1257P	8/3/00	60/223,116
United States	2750-1255P	8/3/00	60/223,098
United States	2750-1053P	8/3/00	60/223,100
United States	2750-0999P	8/4/00	09/633,051
United States	2750-1092P	8/4/00	09/635,643
United States	2750-1078P	8/4/00	09/635,640
United States	2750-1000P	8/4/00	09/633,191
United States	2750-1001P	8/4/00	09/633,239
United States	2750-1047P	8/7/00	60/223,329
United States	2750-1114P	8/9/00	60/224,390
United States	2750-1094P	8/9/00	09/635,642
United States	2750-1002P	8/9/00	09/635,277
United States	2750-1115P	8/9/00	60/224,391
United States	2750-1093P	8/10/00	09/635,641
United States	2750-1005P	8/11/00	09/637,563
United States	2750-1101P	8/11/00	09/637,820
United States	2750-1103P	8/11/00	09/637,564
United States	2750-1003P	8/11/00	09/637,837
United States	2750-1104P	8/11/00	09/637,792
United States	2750-1102P	8/11/00	09/637,565
United States	2750-1004P	8/11/00	09/636,555
United States	2750-1096P	8/11/00	09/637,780
United States	2750-1082P	8/14/00	60/224,517
United States	2750-1083P	8/14/00	60/224,516
United States	2750-1085P	8/15/00	60/225,302
United States	2750-1084P	8/15/00	60/225,303
United States	2750-1095P	8/16/00	09/643,672
United States	2750-1112P	8/16/00	60/225,847
United States	2750-1108P	8/16/00	60/225,848
United States	2750-1106P	8/16/00	60/225,849
United States	2750-1110P	8/16/00	60/225,850
United States	2750-1006P	8/17/00	09/641,198

Country	Attorney No.	Filing Date	Application No.
United States	2750-1007P	8/18/00	09/641,359
United States	2750-1098P	8/18/00	09/643,671
United States	2750-1109P	8/18/00	60/226,323
United States	2750-1111P	8/18/00	60/226,324
United States	2750-1107P	8/18/00	60/226,381
United States	2750-1008P	8/18/00	09/641,375
United States	2750-1009P	8/18/00	09/640,695
United States	2750-1105P	8/18/00	60/226,325
United States	2750-1087P	8/21/00	60/226,725
United States	2750-1086P	8/21/00	60/226,452
United States	2750-1010P	8/23/00	09/643,854
United States	2750-1162P	8/23/00	60/227,024
United States	2750-1123P	8/23/00	60/228,125
United States	2750-1116P	8/23/00	60/228,095
United States	2750-1117P	8/23/00	60/228,094
United States	2750-1118P	8/23/00	60/228,126
United States	2750-1122P	8/23/00	60/228,092
United States	2750-1132P	8/23/00	60/227,778
United States	2750-1131P	8/23/00	60/228,093
United States	2750-1124P	8/23/00	60/228,127
United States	2750-1163P	8/23/00	60/227,026
United States	2750-1097P	8/23/00	09/649,866
United States	2750-1130P	8/24/00	60/227,776
United States	2750-1125P	8/24/00	60/228,091
United States	2750-1129P	8/24/00	60/227,775
United States	2750-2125P	8/25/00	60/228,039
United States	2750-2127P	8/25/00	60/228,032
United States	2750-2128P	8/25/00	60/228,149
United States	2750-1214P	8/25/00	60/228,240
United States	2750-1213P	8/25/00	60/228,221
United States	2750-1212P	8/25/00	60/228,165
United States	2750-1211P	8/25/00	60/228,161
United States	2750-1209P	8/25/00	60/228,054
United States	2750-1215P	8/25/00	60/227,979
United States	2750-1210P	8/25/00	60/228,164
United States	2750-1165P	8/25/00	60/228,046
United States	2750-2123P	8/25/00	60/228,036
United States	2750-2122P	8/25/00	60/228,038
United States	2750-2119P	8/25/00	60/228,027
United States	2750-2118P	8/25/00	60/228,028
United States	2750-2117P	8/25/00	60/228,031
United States	2750-1187P	8/25/00	60/228,177
United States	2750-1224P	8/25/00	
United States	2750-2116P	8/25/00	60/227,793
United States	2750-1166P	8/25/00	60/228,098
United States	2750-1223P	8/25/00	60/228,150
United States	2750-1216P	8/25/00	60/227,954
United States	2750-1167P	8/25/00	60/228,047
United States	2750-1222P	8/25/00	60/228,061
United States	2750-1221P	8/25/00	60/228,187

Country	Attorney No.	Filing Date	Application No.
United States	2750-1220P	8/25/00	60/227,931
United States	2750-1219P	8/25/00	60/228,043
United States	2750-1218P	8/25/00	60/227,929
United States	2750-1217P	8/25/00	60/228,217
United States	2750-2124P	8/25/00	60/227,790
United States	2750-1164P	8/25/00	60/228,163
United States	2750-1194P	8/25/00	60/228,044
United States	2750-1185P	8/25/00	60/228,209
United States	2750-1182P	8/25/00	60/228,153
United States	2750-1183P	8/25/00	60/228,179
United States	2750-1184P	8/25/00	60/228,180
United States	2750-1186P	8/25/00	60/228,178
United States	2750-1188P	8/25/00	60/227,976
United States	2750-1189P	8/25/00	60/228,207
United States	2750-1190P	8/25/00	60/228,048
United States	2750-1191P	8/25/00	60/228,096
United States	2750-1180P	8/25/00	60/228,202
United States	2750-1193P	8/25/00	60/227,936
United States	2750-1179P	8/25/00	60/228,151
United States	2750-1195P	8/25/00	60/228,216
United States	2750-1196P	8/25/00	60/228,065
United States	2750-1197P	8/25/00	60/227,975
United States	2750-1198P	8/25/00	60/228,181
United States	2750-1199P	8/25/00	60/228,063
United States	2750-1200P	8/25/00	60/228,064
United States	2750-2130P	8/25/00	60/227,777
United States	2750-1155P	8/25/00	60/228,224
United States	2750-1128P	8/25/00	60/227,726
United States	2750-2132P	8/25/00	60/227,791
United States	2750-1192P	8/25/00	60/227,932
United States	2750-1168P	8/25/00	60/228,052
United States	2750-1207P	8/25/00	60/227,982
United States	2750-1206P	8/25/00	60/227,978
United States	2750-1205P	8/25/00	60/228,053
United States	2750-1204P	8/25/00	60/227,955
United States	2750-1203P	8/25/00	60/227,939
United States	2750-1202P	8/25/00	60/228,074
United States	2750-1201P	8/25/00	60/228,055
United States	2750-2137P	8/25/00	
United States	2750-2136P	8/25/00	60/228,247
United States	2750-1181P	8/25/00	60/228,208
United States	2750-2133P	8/25/00	60/228,041
United States	2750-1208P	8/25/00	60/228,189
United States	2750-1169P	8/25/00	60/228,049
United States	2750-1170P	8/25/00	60/228,132
United States	2750-1171P	8/25/00	60/228,152
United States	2750-1172P	8/25/00	60/228,135
United States	2750-1173P	8/25/00	60/228,322
United States	2750-1174P	8/25/00	60/228,156
United States	2750-1175P	8/25/00	60/228,323

Country	Attorney No.	Filing Date	Application No.
United States	2750-1176P	8/25/00	60/228,133
United States	2750-1177P	8/25/00	60/228,320
United States	2750-1178P	8/25/00	60/228,159
United States	2750-2135P	8/25/00	60/228,246
United States	2750-1143P	8/25/00	60/227,730
United States	2750-2120P	8/25/00	
United States	2750-1150P	8/25/00	60/227,780
United States	2750-1152P	8/25/00	60/227,774
United States	2750-1151P	8/25/00	60/227,725
United States	2750-1149P	8/25/00	60/227,769
United States	2750-1146P	8/25/00	60/227,773
United States	2750-1147P	8/25/00	60/228,033
United States	2750-1145P	8/25/00	60/227,728
United States	2750-1144P	8/25/00	60/227,770
United States	2750-1156P	8/25/00	60/228,279
United States	2750-1137P	8/25/00	60/227,732
United States	2750-1136P	8/25/00	60/227,731
United States	2750-1135P	8/25/00	60/227,783
United States	2750-1134P	8/25/00	60/227,781
United States	2750-1120P	8/25/00	60/227,779
United States	2750-1013P	8/25/00	09/645,441
United States	2750-2126P	8/25/00	60/228,030
United States	2750-2121P	8/25/00	60/228,026
United States	2750-2129P	8/25/00	60/228,040
United States	2750-1141P	8/25/00	60/227,792
United States	2750-1133P	8/25/00	60/228,025
United States	2750-1126P	8/25/00	60/227,771
United States	2750-1099P	8/25/00	09/649,868
United States	2750-1140P	8/25/00	60/227,734
United States	2750-2131P	8/25/00	60/228,037
United States	2750-1127P	8/25/00	60/227,727
United States	2750-1100P	8/25/00	09/649,867
United States	2750-1148P	8/25/00	60/228,024
United States	2750-1121P	8/25/00	60/227,782
United States	2750-1142P	8/25/00	60/227,733
United States	2750-1138P	8/25/00	60/227,729
United States	2750-1139P	8/25/00	60/228,167
United States	2750-1012P	8/25/00	09/648,708
United States	2750-1011P	8/25/00	09/645,440
United States	2750-2115P	8/25/00	
United States	2750-1119P	8/25/00	60/228,029
United States	2750-1014P	8/30/00	09/651,370
United States	2750-1226P	8/30/00	60/228,897
United States	2750-1225P	8/30/00	60/228,898
United States	2750-1015P	8/31/00	09/653,466
United States	2750-1091P	8/31/00	60/229,520
United States	2750-1113P	8/31/00	60/237,361
United States	2750-1089P	8/31/00	60/237,362
United States	2750-1088P	8/31/00	60/229,521
United States	2750-1090P	8/31/00	60/229,519

Country	Attorney No.	Filing Date	Application No.
United States	2750-1016P	9/1/00	09/654,547
United States	2750-1158P	9/6/00	60/231,837
United States	2750-1228P	9/6/00	60/230,434
United States	2750-1157P	9/6/00	60/231,840
United States	2750-1161P	9/6/00	60/231,834
United States	2750-1159P	9/6/00	60/231,833
United States	2750-1160P	9/6/00	60/231,835
United States	2750-1227P	9/6/00	60/230,430
United States	2750-1017P	9/7/00	09/657,454
United States	2750-1018P	9/8/00	09/657,569
United States	2750-1019P	9/13/00	09/660,883
United States	2750-1229P	9/13/00	60/232,044
United States	2750-1230P	9/13/00	60/232,043
United States	2750-1232P	9/15/00	60/232,865
United States	2750-1231P	9/15/00	60/232,858
United States	2750-1020P	9/15/00	09/663,196
United States	2750-1021P	9/15/00	09/663,195
United States	2750-1234P	9/18/00	60/233,634
United States	2750-1233P	9/18/00	60/233,621
United States	2750-1254P	9/20/00	60/234,178
United States	2750-1253P	9/20/00	60/234,179
United States	2750-1022P	9/20/00	09/665,714
United States	2750-1259P	9/21/00	60/234,233
United States	2750-1261P	9/21/00	60/234,220
United States	2750-1260P	9/21/00	60/234,217
United States	2750-1024P	9/22/00	09/667,517
United States	2750-1025P	9/22/00	09/667,229
United States	2750-1023P	9/22/00	09/667,597
United States	2750-1262P	9/25/00	60/234,968
United States	2750-1263P	9/25/00	60/234,979
United States	2750-1264P	9/25/00	60/234,974
United States	2750-1265P	9/25/00	60/235,118
United States	2750-1266P	9/26/00	60/234,949
United States	2750-1267P	9/27/00	60/235,577
United States	2750-1026P	9/28/00	09/671,635
United States	2750-1268P	9/28/00	60/235,934
United States	2750-1027P	9/29/00	09/672,075
United States	2750-1269P	9/29/00	60/236,380
United States	2750-1270P	10/2/00	60/236,732
United States	2750-1271P	10/2/00	60/237,035
United States	2750-1273P	10/4/00	60/237,505
United States	2750-1272P	10/4/00	60/237,379
United States	2750-1028P	10/4/00	09/679,203
United States	2750-1029P	10/5/00	09/678,223
United States	2750-1274P	10/5/00	60/237,686
United States	2750-1031P	10/6/00	09/680,490
United States	2750-1032P	10/6/00	09/680,498
United States	2750-1030P	10/6/00	09/680,499
United States	2750-1277P	10/10/00	60/238,456
United States	2750-1275P	10/10/00	60/238,473

Country	Attorney No.	Filing Date	Application No.
United States	2750-1276P	10/10/00	60/238,472
United States	2750-1278P	10/10/00	60/238,421
United States	2750-1279P	10/11/00	60/239,091
United States	2750-1280P	10/11/00	60/239,245
United States	2750-1033P	10/12/00	09/686,093
United States	2750-1242P	10/13/00	09/688,051
United States	2750-1240P	10/13/00	09/689,983
United States	2750-1235P	10/13/00	
United States	2750-1236P	10/13/00	
United States	2750-1239P	10/13/00	09/689,982
United States	2750-1238P	10/13/00	09/688,052
United States	2750-1241P	10/13/00	
United States	2750-1237P	10/13/00	09/689,980
United States	2750-1282P	10/17/00	60/240,863
United States	2750-1281P	10/17/00	60/240,862
United States	2750-1034P	10/18/00	09/690,745
United States	2750-1287P	10/19/00	09/691,044
United States	2750-1285P	10/19/00	09/691,039
United States	2750-1288P	10/19/00	
United States	2750-1290P	10/19/00	09/691,056
United States	2750-1291P	10/19/00	09/691,038
United States	2750-1292P	10/19/00	09/691,031
United States	2750-1294P	10/19/00	09/691,018
United States	2750-1284P	10/19/00	60/241,367
United States	2750-1283P	10/19/00	60/241,368
United States	2750-1293P	10/19/00	09/691,019
United States	2750-1286P	10/19/00	09/691,020
United States	2750-1289P	10/19/00	09/691,045
United States	2750-1295P	10/20/00	09/692,157
United States	2750-1301P	10/20/00	09/692,108
United States	2750-1302P	10/20/00	09/692,152
United States	2750-1303P	10/20/00	09/692,696
United States	2750-1297P	10/20/00	09/692,714
United States	2750-1300P	10/20/00	09/692,717
United States	2750-1299P	10/20/00	09/692,148
United States	2750-1296P	10/20/00	09/692,154
United States	2750-1305P	10/20/00	60/241,750
United States	2750-1304P	10/20/00	60/241,751
United States	2750-1298P	10/20/00	09/692,153
United States	2750-1306P	10/23/00	60/242,065
United States	2750-1307P	10/23/00	60/242,072
United States	2750-1320P	10/24/00	60/242,686
United States	2750-1308P	10/25/00	09/695,391
United States	2750-1321P	10/25/00	60/242,705
United States	2750-1310P	10/25/00	09/695,387
United States	2750-1322P	10/25/00	60/242,706
United States	2750-1309P	10/25/00	09/696,751
United States	2750-1311P	10/26/00	09/696,305
United States	2750-1324P	10/26/00	60/243,288
United States	2750-1323P	10/26/00	60/243,289

Country	Attorney No.	Filing Date	Application No.
United States	2750-1313P	10/26/00	09/696,017
United States	2750-1312P	10/26/00	09/696,284
United States	2750-1326P	10/27/00	60/243,478
United States	2750-1318P	10/27/00	09/697,081
United States	2750-1315P	10/27/00	09/697,056
United States	2750-1314P	10/27/00	09/697,080
United States	2750-1317P	10/27/00	09/697,718
United States	2750-1316P	10/27/00	09/697,145
United States	2750-1319P	10/27/00	09/697,076
United States	2750-1325P	10/27/00	60/243,398
United States	2750-1328P	10/30/00	60/243,735
United States	2750-1327P	10/30/00	60/243,723
United States	2750-1330P	11/1/00	09/702,841
United States	2750-1331P	11/1/00	09/702,873
United States	2750-1341P	11/1/00	60/244,691
United States	2750-1342P	11/1/00	60/244,747
United States	2750-1329P	11/1/00	09/702,840
United States	2750-1333P	11/2/00	09/703,627
United States	2750-1334P	11/2/00	09/703,619
United States	2750-1332P	11/2/00	09/703,932
United States	2750-1344P	11/2/00	60/244,920
United States	2750-1343P	11/2/00	60/244,923
United States	2750-1340P	11/3/00	09/704,540
United States	2750-1339P	11/3/00	09/704,541
United States	2750-1336P	11/3/00	09/704,550
United States	2750-1335P	11/3/00	09/704,559
United States	2750-1337P	11/3/00	09/704,836
United States	2750-1346P	11/3/00	60/245,165
United States	2750-1345P	11/3/00	60/245,164
United States	2750-1338P	11/3/00	09/704,542
United States	2750-1360P	11/6/00	60/245,576
United States	2750-1359P	11/6/00	60/245,676
United States	2750-1347P	11/8/00	09/708,092
United States	2750-1243P	11/9/00	09/708,427
United States	2750-1244P	11/9/00	60/246,732
United States	2750-1247P	11/13/00	60/247,050
United States	2750-1248P	11/13/00	60/247,049
United States	2750-1246P	11/13/00	60/247,051
United States	2750-1245P	11/13/00	60/247,010
United States	2750-1348P	11/15/00	60/248,198
United States	2750-1349P	11/15/00	60/248,197
United States	2750-1350P	11/16/00	60/248,555
United States	2750-1352P	11/17/00	60/249,257
United States	2750-1351P	11/17/00	60/249,256
United States	2750-1354P	11/20/00	60/249,453
United States	2750-1353P	11/20/00	60/249,454
United States	2750-1355P	11/21/00	60/252,080
United States	2750-1357P	11/22/00	60/252,465
United States	2750-1356P	11/22/00	60/252,464
United States	2750-1358P	11/24/00	60/252,598

Country	Attorney No.	Filing Date	Application No.
United States	2750-1361P	11/24/00	60/252,590
United States	2750-1362P	11/28/00	60/253,140
United States	2750-1363P	11/29/00	60/253,722
United States	2750-1364P	11/29/00	60/253,748
United States	2750-1249P	12/1/00	09/726,578
United States	2750-1365P	12/1/00	60/250,356
United States	2750-1366P	12/4/00	60/250,464
United States	2750-1367P	12/6/00	60/251,387
United States	2750-1369P	12/7/00	60/251,508
United States	2750-1368P	12/7/00	60/251,504
United States	2750-1371P	12/8/00	60/251,854
United States	2750-1250P	12/8/00	09/731,809
United States	2750-1370P	12/8/00	60/251,853
United States	2750-1372P	12/11/00	60/254,174
United States	2750-1373P	12/11/00	60/254,196
United States	2750-1374P	12/13/00	60/254,891
United States	2750-1375P	12/15/00	60/256,503
United States	2750-1376P	12/15/00	60/255,415
United States	2750-1377P	12/18/00	60/255,891
United States	2750-1378P	12/18/00	60/255,892
United States	2750-1379P	12/19/00	60/256,306
United States	2750-1381P	12/21/00	60/256,929
United States	2750-1380P	12/21/00	09/741,043
United States	2750-1382P	12/27/00	60/257,978
United States	2750-1383P	12/29/00	09/750,044
United States	2750-1385P	1/2/01	09/750,910
United States	2750-1384P	1/2/01	60/258,880
United States	2750-1386P	1/3/01	09/752,823
United States	2750-1154P	1/5/01	09/754,185
United States	2750-1153P	1/5/01	09/754,184
United States	2750-1388P	1/19/01	60/262,389
United States	2750-1389P	1/19/01	60/262,359
United States	2750-1387P	1/19/01	09/764,425
United States	2750-1392P	1/26/01	60/264,027
United States	2750-1390P	1/26/01	09/769,525
United States	2750-1391P	1/26/01	60/264,026
United States	2750-1394P	1/29/01	60/264,257
United States	2750-1393P	1/29/01	60/264,282
United States	2750-1395P	1/31/01	
United States	2750-1397P	1/31/01	09/774,090
United States	2750-1396P	1/31/01	
United States	2750-1398P	1/31/01	
United States	2750-1401P	2/1/01	
United States	2750-1400P	2/1/01	
United States	2750-1399P	2/1/01	
United States	2750-1403P	2/6/01	
United States	2750-1402P	2/6/01	
United States	2750-1404P	2/7/01	
United States	2750-1405P	2/8/01	
United States	2750-1406P	2/9/01	

Country	Attorney No.	Filing Date	Application No.
United States	2750-1408P	2/9/01	
United States	2750-1407P	2/9/01	
United States	2750-1409P	2/12/01	
United States	2750-1410P	2/12/01	
United States	2750-1412P	2/14/01	
United States	2750-1411P	2/15/01	
United States	2750-1413P	2/16/01	
United States	2750-1414P	2/21/01	
United States	2750-1415P	2/21/01	
United States	2750-1417P	2/21/01	
United States	2750-1416P	2/21/01	
United States	2750-1418P	2/22/01	
United States	2750-1419P	2/23/01	
United States	2750-1420P	2/26/01	
United States	2750-1421P	2/26/01	
United States	2750-1425P	2/28/01	
United States	2750-1424P	2/28/01	
United States	2750-1422P	3/1/01	
United States	2750-1423P	3/1/01	
United States	2750-1427P	3/2/01	
United States	2750-1430P	3/7/01	
United States	2750-1431P	3/7/01	
United States	2750-1429P	3/7/01	
United States	2750-1428P	3/8/01	
United States	2750-1432P	3/16/01	

All applications listed in the table above are expressly incorporated herein by reference.

The SDFs of the invention can also be used as probes to search for genes that are related to the SDF within a species. Such related genes are typically considered to be members of a gene family. In such a case, the sequence similarity will often be concentrated into one or a few fragments of the sequence. The fragments of similar sequence that define the gene family typically encode a fragment of a protein or RNA that has an enzymatic or structural function. The percentage of identity in the amino acid sequence of the domain that defines the gene family is preferably at least 70%, more preferably 80 to 95%, most preferably 85 to 99%. To search for members of a gene family within a species, a low stringency hybridization is usually performed, but this will depend upon the size, distribution and degree of sequence divergence of domains that define the gene family. SDFs encompassing regulatory regions can be used to identify coordinately expressed genes by using the regulatory region sequence of the SDF as a probe.

In the instances where the SDFs are identified as being expressed from genes that confer a particular phenotype, then the SDFs can also be used as probes to assay plants of different species for those phenotypes.

#### I.C. Methods to Inhibit Gene Expression

5 The nucleic acid molecules of the present invention can be used to inhibit gene transcription and/or translation. Example of such methods include, without limitation:

Antisense Constructs;

Ribozyme Constructs;

Chimeroplast Constructs;

10 Co-Suppression;

Transcriptional Silencing; and

Other Methods of Gene Expression.

##### 15 C.1 Antisense

In some instances it is desirable to suppress expression of an endogenous or exogenous gene. A well-known instance is the FLAVOR-SAVOR™ tomato, in which the gene encoding ACC synthase is inactivated by an antisense approach, thus delaying softening of the fruit after ripening. See for example, U.S. Patent No. 5,859,330; U.S. Patent No. 5,723,766; Oeller, et al, *Science*, 254:437-439(1991); and Hamilton et al, *Nature*, 346:284-287 (1990). Also, timing of flowering can be controlled by suppression of the *FLOWERING LOCUS C (FLC)*; high levels of this transcript are associated with late flowering, while absence of *FLC* is associated with early flowering (S.D. Michaels et al., *Plant Cell* 11:949 (1999). Also, the transition of apical meristem from production of leaves with associated shoots to flowering is regulated by *TERMINAL FLOWER1*, *APETALA1* and *LEAFY*. Thus, when it is desired to induce a transition from shoot production to flowering, it is desirable to suppress *TFL1* expression (S.J. Liljegren, *Plant Cell* 11:1007 (1999)). As another instance, arrested ovule development and female sterility result from suppression of the ethylene forming enzyme but can be reversed by application of ethylene (D. De Martinis et al., *Plant Cell* 11:1061 (1999)). The ability to manipulate female fertility of plants is useful in increasing fruit production and creating hybrids.

In the case of polynucleotides used to inhibit expression of an endogenous gene, the introduced sequence need not be perfectly identical to a sequence of the target endogenous

gene. The introduced polynucleotide sequence will typically be at least substantially identical to the target endogenous sequence.

Some polynucleotide SDFs in Table 1 and Table 2, if present represent sequences that are expressed in corn, wheat, rice, soybean *Arabidopsis* and/or other plants. Thus the invention includes using these sequences to generate antisense constructs to inhibit translation and/or degradation of transcripts of said SDFs, typically in a plant cell.

To accomplish this, a polynucleotide segment from the desired gene that can hybridize to the mRNA expressed from the desired gene (the "antisense segment") is operably linked to a promoter such that the antisense strand of RNA will be transcribed when the construct is present in a host cell. A regulated promoter can be used in the construct to control transcription of the antisense segment so that transcription occurs only under desired circumstances.

The antisense segment to be introduced generally will be substantially identical to at least a fragment of the endogenous gene or genes to be repressed. The sequence, however, need not be perfectly identical to inhibit expression. Further, the antisense product may hybridize to the untranslated region instead of or in addition to the coding sequence of the gene. The vectors of the present invention can be designed such that the inhibitory effect applies to other proteins within a family of genes exhibiting homology or substantial homology to the target gene.

For antisense suppression, the introduced antisense segment sequence also need not be full length relative to either the primary transcription product or the fully processed mRNA. Generally, a higher percentage of sequence identity can be used to compensate for the use of a shorter sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and homology of non-coding segments may be equally effective. Normally, a sequence of between about 30 or 40 nucleotides and the full length of the transcript can be used, though a sequence of at least about 100 nucleotides is preferred, a sequence of at least about 200 nucleotides is more preferred, and a sequence of at least about 500 nucleotides is especially preferred.

### 30 C.2. Ribozymes

It is also contemplated that gene constructs representing ribozymes and based on the SDFs in Table 1 and Table 2, if present are an object of the invention. Ribozymes can also be used to inhibit expression of genes by suppressing the translation of the mRNA into a

polypeptide. It is possible to design ribozymes that specifically pair with virtually any target RNA and cleave the phosphodiester backbone at a specific location, thereby functionally inactivating the target RNA. In carrying out this cleavage, the ribozyme is not itself altered, and is thus capable of recycling and cleaving other molecules, making it a true enzyme. The 5 inclusion of ribozyme sequences within antisense RNAs confers RNA-cleaving activity upon them, thereby increasing the activity of the constructs.

A number of classes of ribozymes have been identified. One class of ribozymes is derived from a number of small circular RNAs, which are capable of self-cleavage and replication in plants. The RNAs replicate either alone (viroid RNAs) or with a helper virus 10 (satellite RNAs). Examples include RNAs from avocado sunblotch viroid and the satellite RNAs from tobacco ringspot virus, lucerne transient streak virus, velvet tobacco mottle virus, solanum nodiflorum mottle virus and subterranean clover mottle virus. The design and use of target RNA-specific ribozymes is described in Haseloff et al. *Nature*, 334:585 (1988).

15 Like the antisense constructs above, the ribozyme sequence fragment necessary for pairing need not be identical to the target nucleotides to be cleaved, nor identical to the sequences in Table 1 and Table 2, if present. Ribozymes may be constructed by combining the ribozyme sequence and some fragment of the target gene which would allow recognition of the target gene mRNA by the resulting ribozyme molecule. Generally, the sequence in 20 the ribozyme capable of binding to the target sequence exhibits a percentage of sequence identity with at least 80%, preferably with at least 85%, more preferably with at least 90% and most preferably with at least 95%, even more preferably, with at least 96%, 97%, 98% or 99% sequence identity to some fragment of a sequence in Table 1 and Table 2, if present or the complement thereof. The ribozyme can be equally effective in inhibiting mRNA translation 25 by cleaving either in the untranslated or coding regions. Generally, a higher percentage of sequence identity can be used to compensate for the use of a shorter sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and homology of non-coding segments may be equally effective.

### C.3. Chimeroplasts

30 The SDFs of the invention, such as those described by Table 1 and Table 2, if present, can also be used to construct chimeroplasts that can be introduced into a cell to produce at least one specific nucleotide change in a sequence corresponding to the SDF of the invention. A chimeroplast is an oligonucleotide comprising DNA and/or RNA that

specifically hybridizes to a target region in a manner which creates a mismatched base-pair. This mismatched base-pair signals the cell's repair enzyme machinery which acts on the mismatched region resulting in the replacement, insertion or deletion of designated nucleotide(s). The altered sequence is then expressed by the cell's normal cellular mechanisms. Chimeroplasts can be designed to repair mutant genes, modify genes, introduce site-specific mutations, and/or act to interrupt or alter normal gene function (US Pat. Nos. 6,010,907 and 6,004,804; and PCT Pub. No. WO99/58723 and WO99/07865).

#### C.4. Sense Suppression

The SDFs of Table 1 and Table 2, if present of the present invention are also useful to modulate gene expression by sense suppression. Sense suppression represents another 10 method of gene suppression by introducing at least one exogenous copy or fragment of the endogenous sequence to be suppressed.

Introduction of expression cassettes in which a nucleic acid is configured in the sense orientation with respect to the promoter into the chromosome of a plant or by a self-replicating virus has been shown to be an effective means by which to induce degradation of mRNAs of 15 target genes. For an example of the use of this method to modulate expression of endogenous genes see, Napoli et al., *The Plant Cell* 2:279 (1990), and U.S. Patents Nos. 5,034,323, 5,231,020, and 5,283,184. Inhibition of expression may require some transcription of the introduced sequence.

For sense suppression, the introduced sequence generally will be substantially identical 20 to the endogenous sequence intended to be inactivated. The minimal percentage of sequence identity will typically be greater than about 65%, but a higher percentage of sequence identity might exert a more effective reduction in the level of normal gene products. Sequence identity of more than about 80% is preferred, though about 95% to absolute identity would be most preferred. As with antisense regulation, the effect would likely apply to any other proteins 25 within a similar family of genes exhibiting homology or substantial homology to the suppressing sequence.

#### C.5. Transcriptional Silencing

The nucleic acid sequences of the invention, including the SDFs of Table 1 and Table 30 2, if present, and fragments thereof, contain sequences that can be inserted into the genome of an organism resulting in transcriptional silencing. Such regulatory sequences need not be operatively linked to coding sequences to modulate transcription of a gene. Specifically, a

promoter sequence without any other element of a gene can be introduced into a genome to transcriptionally silence an endogenous gene (see, for example, Vaucheret, H et al. (1998) The Plant Journal 16: 651-659). As another example, triple helices can be formed using oligonucleotides based on sequences from Table 1 and Table 2, if present, fragments thereof, and substantially similar sequence thereto. The oligonucleotide can be delivered to the host cell and can bind to the promoter in the genome to form a triple helix and prevent transcription. An oligonucleotide of interest is one that can bind to the promoter and block binding of a transcription factor to the promoter. In such a case, the oligonucleotide can be complementary to the sequences of the promoter that interact with transcription binding factors.

10

#### 25 C.6. Other Methods to Inhibit Gene Expression

Yet another means of suppressing gene expression is to insert a polynucleotide into the gene of interest to disrupt transcription or translation of the gene.

15 Low frequency homologous recombination can be used to target a polynucleotide insert to a gene by flanking the polynucleotide insert with sequences that are substantially similar to the gene to be disrupted. Sequences from Table 1 and Table 2, if present, fragments thereof, and substantially similar sequence thereto can be used for homologous recombination.

20 In addition, random insertion of polynucleotides into a host cell genome can also be used to disrupt the gene of interest. Azpiroz-Leehan et al., *Trends in Genetics* 13:152 (1997). In this method, screening for clones from a library containing random insertions is preferred to identifying those that have polynucleotides inserted into the gene of interest. Such screening can be performed using probes and/or primers described above based on sequences from Table 1 and Table 2, if present, fragments thereof, and substantially similar sequence thereto. The screening can also be performed by selecting clones or R<sub>1</sub> plants having a desired phenotype.

#### 25 I.D. Methods of Functional Analysis

The constructs described in the methods under I.C. above can be used to determine the function of the polypeptide encoded by the gene that is targeted by the constructs.

30 Down-regulating the transcription and translation of the targeted gene in the host cell or organisms, such as a plant, may produce phenotypic changes as compared to a wild-type cell or organism. In addition, *in vitro* assays can be used to determine if any biological

activity, such as calcium flux, DNA transcription, nucleotide incorporation, etc., are being modulated by the down-regulation of the targeted gene.

Coordinated regulation of sets of genes, e.g., those contributing to a desired polygenic trait, is sometimes necessary to obtain a desired phenotype. SDFs of the invention representing transcription activation and DNA binding domains can be assembled into hybrid transcriptional activators. These hybrid transcriptional activators can be used with their corresponding DNA elements (i.e., those bound by the DNA-binding SDFs) to effect coordinated expression of desired genes (J.J. Schwarz et al., *Mol. Cell. Biol.* 12:266 (1992), A. Martinez et al., *Mol. Gen. Genet.* 261:546 (1999)).

The SDFs of the invention can also be used in the two-hybrid genetic systems to identify networks of protein-protein interactions (L. McAlister-Henn et al., *Methods* 19:330 (1999), J.C. Hu et al., *Methods* 20:80 (2000), M. Golovkin et al., *J. Biol. Chem.* 274:36428 (1999), K. Ichimura et al., *Biochem. Biophys. Res. Comm.* 253:532 (1998)). The SDFs of the invention can also be used in various expression display methods to identify important protein-DNA interactions (e.g. B. Luo et al., *J. Mol. Biol.* 266:479 (1997)).

#### I.E. Promoters

The SDFs of the invention are also useful as structural or regulatory sequences in a construct for modulating the expression of the corresponding gene in a plant or other organism, e.g. a symbiotic bacterium. For example, promoter sequences associated to SDFs of Table 1 and Table 2, if present of the present invention can be useful in directing expression of coding sequences either as constitutive promoters or to direct expression in particular cell types, tissues, or organs or in response to environmental stimuli.

With respect to the SDFs of the present invention a promoter is likely to be a relatively small portion of a genomic DNA (gDNA) sequence located in the first 2000 nucleotides upstream from an initial exon identified in a gDNA sequence or initial “ATG” or methionine codon or translational start site in a corresponding cDNA sequence. Such promoters are more likely to be found in the first 1000 nucleotides upstream of an initial ATG or methionine codon or translational start site of a cDNA sequence corresponding to a gDNA sequence. In particular, the promoter is usually located upstream of the transcription start site. The fragments of a particular gDNA sequence that function as elements of a promoter in a plant cell will preferably be found to hybridize to gDNA sequences presented and described in Table

1 and Table 2, if present at medium or high stringency, relevant to the length of the probe and its base composition.

Promoters are generally modular in nature. Promoters can consist of a basal promoter that functions as a site for assembly of a transcription complex comprising an RNA polymerase, for example RNA polymerase II. A typical transcription complex will include additional factors such as TF<sub>II</sub>B, TF<sub>II</sub>D, and TF<sub>II</sub>E. Of these, TF<sub>II</sub>D appears to be the only one to bind DNA directly. The promoter might also contain one or more enhancers and/or suppressors that function as binding sites for additional transcription factors that have the function of modulating the level of transcription with respect to tissue specificity and of transcriptional responses to particular environmental or nutritional factors, and the like.

Short DNA sequences representing binding sites for proteins can be separated from each other by intervening sequences of varying length. For example, within a particular functional module, protein binding sites may be constituted by regions of 5 to 60, preferably 10 to 30, more preferably 10 to 20 nucleotides. Within such binding sites, there are typically 2 to 6 nucleotides that specifically contact amino acids of the nucleic acid binding protein. The protein binding sites are usually separated from each other by 10 to several hundred nucleotides, typically by 15 to 150 nucleotides, often by 20 to 50 nucleotides. DNA binding sites in promoter elements often display dyad symmetry in their sequence. Often elements binding several different proteins, and/or a plurality of sites that bind the same protein, will be combined in a region of 50 to 1,000 basepairs.

Elements that have transcription regulatory function can be isolated from their corresponding endogenous gene, or the desired sequence can be synthesized, and recombined in constructs to direct expression of a coding region of a gene in a desired tissue-specific, temporal-specific or other desired manner of inducibility or suppression. When hybridizations are performed to identify or isolate elements of a promoter by hybridization to the long sequences presented in Table 1 and Table 2, if present, conditions are adjusted to account for the above-described nature of promoters. For example short probes, constituting the element sought, are preferably used under low temperature and/or high salt conditions. When long probes, which might include several promoter elements are used, low to medium stringency conditions are preferred when hybridizing to promoters across species.

If a nucleotide sequence of an SDF, or part of the SDF, functions as a promoter or fragment of a promoter, then nucleotide substitutions, insertions or deletions that do not substantially affect the binding of relevant DNA binding proteins would be considered equivalent to the exemplified nucleotide sequence. It is envisioned that there are instances

where it is desirable to decrease the binding of relevant DNA binding proteins to silence or down-regulate a promoter, or conversely to increase the binding of relevant DNA binding proteins to enhance or up-regulate a promoter and vice versa. In such instances, 5 polynucleotides representing changes to the nucleotide sequence of the DNA-protein contact region by insertion of additional nucleotides, changes to identity of relevant nucleotides, including use of chemically-modified bases, or deletion of one or more nucleotides are considered encompassed by the present invention. In addition, fragments of the promoter sequences described by Table 1 and Table 2, if present and variants thereof can be fused with other promoters or fragments to facilitate transcription and/or 10 transcription in specific type of cells or under specific conditions.

Promoter function can be assayed by methods known in the art, preferably by measuring activity of a reporter gene operatively linked to the sequence being tested for promoter function. Examples of reporter genes include those encoding luciferase, green fluorescent protein, GUS, neo, cat and bar.

15 I.F. UTRs and Junctions

Polynucleotides comprising untranslated (UTR) sequences and intron/exon junctions are also within the scope of the invention. UTR sequences include introns and 5' or 3' untranslated regions ( 5' UTRs or 3' UTRs). Fragments of the sequences shown in Table 1 and Table 2, if present can comprise UTRs and intron/exon junctions.

20 These fragments of SDFs, especially UTRs, can have regulatory functions related to, for example, translation rate and mRNA stability. Thus, these fragments of SDFs can be isolated for use as elements of gene constructs for regulated production of polynucleotides encoding desired polypeptides.

Introns of genomic DNA segments might also have regulatory functions. 25 Sometimes regulatory elements, especially transcription enhancer or suppressor elements, are found within introns. Also, elements related to stability of heteronuclear RNA and efficiency of splicing and of transport to the cytoplasm for translation can be found in intron elements. Thus, these segments can also find use as elements of expression vectors intended for use to transform plants.

30 Just as with promoters UTR sequences and intron/exon junctions can vary from those shown in Table 1 and Table 2, if present. Such changes from those sequences preferably will not affect the regulatory activity of the UTRs or intron/exon junction sequences on expression, transcription, or translation unless selected to do so. However, in

some instances, down- or up-regulation of such activity may be desired to modulate traits or phenotypic or *in vitro* activity.

#### I.G. Coding Sequences

Isolated polynucleotides of the invention can include coding sequences that encode 5 polypeptides comprising an amino acid sequence encoded by sequences in Table 1 and Table 2, if present or an amino acid sequence presented in Table 1 and Table 2, if present.

A nucleotide sequence encodes a polypeptide if a cell (or a cell free *in vitro* system) expressing that nucleotide sequence produces a polypeptide having the recited amino acid sequence when the nucleotide sequence is transcribed and the primary transcript is 10 subsequently processed and translated by a host cell (or a cell free *in vitro* system) harboring the nucleic acid. Thus, an isolated nucleic acid that encodes a particular amino acid sequence can be a genomic sequence comprising exons and introns or a cDNA sequence that represents the product of splicing thereof. An isolated nucleic acid encoding an amino acid sequence also encompasses heteronuclear RNA, which contains sequences 15 that are spliced out during expression, and mRNA, which lacks those sequences.

Coding sequences can be constructed using chemical synthesis techniques or by isolating coding sequences or by modifying such synthesized or isolated coding sequences as described above.

In addition to coding sequences encoding the polypeptide sequences of Table 1 and 20 Table 2, if present, which are native to corn, *Arabidopsis*, soybean, rice, wheat, and other plants the isolated polynucleotides can be polynucleotides that encode variants, fragments, and fusions of those native proteins. Such polypeptides are described below in part II.

In variant polynucleotides generally, the number of substitutions, deletions or 25 insertions is preferably less than 20%, more preferably less than 15%; even more preferably less than 10%, 5%, 3% or 1% of the number of nucleotides comprising a particularly exemplified sequence. It is generally expected that non-degenerate nucleotide sequence changes that result in 1 to 10, more preferably 1 to 5 and most preferably 1 to 3 amino acid 30 insertions, deletions or substitutions will not greatly affect the function of an encoded polypeptide. The most preferred embodiments are those wherein 1 to 20, preferably 1 to 10, most preferably 1 to 5 nucleotides are added to, deleted from and/or substituted in the sequences specifically disclosed in Table 1 and Table 2, if present.

Insertions or deletions in polynucleotides intended to be used for encoding a polypeptide preferably preserve the reading frame. This consideration is not so important in instances when the polynucleotide is intended to be used as a hybridization probe.

## II. Polypeptides and Proteins

### 5 IIA. Native polypeptides and proteins

10 Polypeptides within the scope of the invention include both native proteins as well as variants, fragments, and fusions thereof. Polypeptides of the invention are those encoded by any of the six reading frames of sequences shown in Table 1 and Table 2, if present, preferably encoded by the three frames reading in the 5' to 3' direction of the sequences as shown.

Native polypeptides include the proteins encoded by the sequences shown in Table 1 and Table 2, if present. Such native polypeptides include those encoded by allelic variants.

15 Polypeptide and protein variants will exhibit at least 75% sequence identity to those native polypeptides of Table 1 and Table 2, if present. More preferably, the polypeptide variants will exhibit at least 85% sequence identity; even more preferably, at least 90% sequence identity; more preferably at least 95%, 96%, 97%, 98%, or 99% sequence identity. Fragments of polypeptide or fragments of polypeptides will exhibit similar percentages of sequence identity to the relevant fragments of the native polypeptide. Fusions will exhibit a similar percentage of sequence identity in that fragment of the fusion represented by the variant of the native peptide.

20 Furthermore, polypeptide variants will exhibit at least one of the functional properties of the native protein. Such properties include, without limitation, protein interaction, DNA interaction, biological activity, immunological activity, receptor binding, signal transduction, transcription activity, growth factor activity, secondary structure, three-dimensional structure, etc. As to properties related to *in vitro* or *in vivo* activities, the variants preferably exhibit at least 60% of the activity of the native protein; more preferably at least 70%, even more preferably at least 80%, 85%, 90% or 95% of at least one activity of the native protein.

30 One type of variant of native polypeptides comprises amino acid substitutions, deletions and/or insertions. Conservative substitutions are preferred to maintain the function or activity of the polypeptide.

Within the scope of percentage of sequence identity described above, a polypeptide of the invention may have additional individual amino acids or amino acid sequences inserted

into the polypeptide in the middle thereof and/or at the N-terminal and/or C-terminal ends thereof. Likewise, some of the amino acids or amino acid sequences may be deleted from the polypeptide.

#### A.1 Antibodies

Isolated polypeptides can be utilized to produce antibodies. Polypeptides of the invention can generally be used, for example, as antigens for raising antibodies by known techniques. The resulting antibodies are useful as reagents for determining the distribution of the antigen protein within the tissues of a plant or within a cell of a plant. The antibodies are also useful for examining the production level of proteins in various tissues, for example in a wild-type plant or following genetic manipulation of a plant, by methods such as Western blotting.

Antibodies of the present invention, both polyclonal and monoclonal, may be prepared by conventional methods. In general, the polypeptides of the invention are first used to immunize a suitable animal, such as a mouse, rat, rabbit, or goat. Rabbits and goats are preferred for the preparation of polyclonal sera due to the volume of serum obtainable, and the availability of labeled anti-rabbit and anti-goat antibodies as detection reagents. Immunization is generally performed by mixing or emulsifying the protein in saline, preferably in an adjuvant such as Freund's complete adjuvant, and injecting the mixture or emulsion parenterally (generally subcutaneously or intramuscularly). A dose of 50-200 µg/injection is typically sufficient. Immunization is generally boosted 2-6 weeks later with one or more injections of the protein in saline, preferably using Freund's incomplete adjuvant. One may alternatively generate antibodies by *in vitro* immunization using methods known in the art, which for the purposes of this invention is considered equivalent to *in vivo* immunization.

Polyclonal antisera is obtained by bleeding the immunized animal into a glass or plastic container, incubating the blood at 25°C for one hour, followed by incubating the blood at 4°C for 2-18 hours. The serum is recovered by centrifugation (e.g., 1,000xg for 10 minutes). About 20-50 ml per bleed may be obtained from rabbits.

Monoclonal antibodies are prepared using the method of Kohler and Milstein, *Nature* 256: 495 (1975), or modification thereof. Typically, a mouse or rat is immunized as described above. However, rather than bleeding the animal to extract serum, the spleen (and optionally several large lymph nodes) is removed and dissociated into single cells. If desired, the spleen cells can be screened (after removal of nonspecifically adherent cells) by applying a cell suspension to a plate, or well, coated with the protein antigen. B-cells producing membrane-

bound immunoglobulin specific for the antigen bind to the plate, and are not rinsed away with the rest of the suspension. Resulting B-cells, or all dissociated spleen cells, are then induced to fuse with myeloma cells to form hybridomas, and are cultured in a selective medium (e.g., hypoxanthine, aminopterin, thymidine medium, "HAT"). The resulting hybridomas are plated by limiting dilution, and are assayed for the production of antibodies which bind specifically to the immunizing antigen (and which do not bind to unrelated antigens). The selected Mab-secreting hybridomas are then cultured either *in vitro* (e.g., in tissue culture bottles or hollow fiber reactors), or *in vivo* (as ascites in mice).

Other methods for sustaining antibody-producing B-cell clones, such as by EBV transformation, are known.

If desired, the antibodies (whether polyclonal or monoclonal) may be labeled using conventional techniques. Suitable labels include fluorophores, chromophores, radioactive atoms (particularly  $^{32}\text{P}$  and  $^{125}\text{I}$ ), electron-dense reagents, enzymes, and ligands having specific binding partners. Enzymes are typically detected by their activity. For example, horseradish peroxidase is usually detected by its ability to convert 3,3',5,5'-tetramethylbenzidine (TNB) to a blue pigment, quantifiable with a spectrophotometer.

#### A.2 In Vitro Applications of Polypeptides

Some polypeptides of the invention will have enzymatic activities that are useful *in vitro*. For example, the soybean trypsin inhibitor (Kunitz) family is one of the numerous families of proteinase inhibitors. It comprises plant proteins which have inhibitory activity against serine proteinases from the trypsin and subtilisin families, thiol proteinases and aspartic proteinases. Thus, these peptides find *in vitro* use in protein purification protocols and perhaps in therapeutic settings requiring topical application of protease inhibitors.

Delta-aminolevulinic acid dehydratase (EC 4.2.1.24) (ALAD) catalyzes the second step in the biosynthesis of heme, the condensation of two molecules of 5-aminolevulinate to form porphobilinogen and is also involved in chlorophyll biosynthesis (Kaczor et al. (1994) Plant Physiol. 1-4: 1411-7; Smith (1988) Biochem. J. 249: 423-8; Schneider (1976) Z. naturforsch. [C] 31: 55-63). Thus, ALAD proteins can be used as catalysts in synthesis of heme derivatives. Enzymes of biosynthetic pathways generally can be used as catalysts for *in vitro* synthesis of the compounds representing products of the pathway.

Polypeptides encoded by SDFs of the invention can be engineered to provide purification reagents to identify and purify additional polypeptides that bind to them. This

allows one to identify proteins that function as multimers or elucidate signal transduction or metabolic pathways. In the case of DNA binding proteins, the polypeptide can be used in a similar manner to identify the DNA determinants of specific binding (S. Pierrou et al., *Anal. Biochem.* 229:99 (1995), S. Chusacultanachai et al., *J. Biol. Chem.* 274:23591 (1999), Q.

5 Lin et al., *J. Biol. Chem.* 272:27274 (1997)).

## II.B . POLYPEPTIDE VARIANTS , FRAGMENTS, AND FUSIONS

Generally, variants , fragments, or fusions of the polypeptides encoded by the SDFs of the invention can exhibit at least one of the activities of the identified domains and/or related polypeptides described in Table 1 and Table 2, if present corresponding to the SDF of interest.

### 10 II.B .(1) Variants

A type of variant of the native polypeptides comprises amino acid substitutions. Conservative substitutions, described above (see II.), are preferred to maintain the function or activity of the polypeptide. Such substitutions include conservation of charge, polarity, hydrophobicity, size, etc. For example, one or more amino acid residues within the sequence 15 can be substituted with another amino acid of similar polarity that acts as a functional equivalent, for example providing a hydrogen bond in an enzymatic catalysis. Substitutes for an amino acid within an exemplified sequence are preferably made among the members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. 20 The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

Within the scope of percentage of sequence identity described above, a polypeptide of the invention may have additional individual amino acids or amino acid sequences inserted 25 into the polypeptide in the middle thereof and/or at the N-terminal and/or C-terminal ends thereof. Likewise, some of the amino acids or amino acid sequences may be deleted from the polypeptide. Amino acid substitutions may also be made in the sequences; conservative substitutions being preferred.

One preferred class of variants are those that comprise (1) the domain of an 30 encoded polypeptide and/or (2) residues conserved between the encoded polypeptide and related polypeptides. For this class of variants, the encoded polypeptide sequence is

changed by insertion, deletion, or substitution at positions flanking the domain and/or conserved residues.

Another class of variants includes those that comprise an encoded polypeptide sequence that is changed in the domain or conserved residues by a conservative substitution.

Yet another class of variants includes those that lack one of the *in vitro* activities, or structural features of the encoded polypeptides. One example is polypeptides or proteins produced from genes comprising dominant negative mutations. Such a variant may comprise an encoded polypeptide sequence with non-conservative changes in a particular domain or group of conserved residues.

#### II.A.(2) FRAGMENTS

Fragments of particular interest are those that comprise a domain identified for a polypeptide encoded by an SDF of the instant invention and variants thereof. Also, fragments that comprise at least one region of residues conserved between an SDF encoded polypeptide and its related polypeptides are of great interest. Fragments are sometimes useful as polypeptides corresponding to genes comprising dominant negative mutations are.

#### II.A.(3)FUSIONS

Of interest are chimeras comprising (1) a fragment of the SDF encoded polypeptide or variants thereof of interest and (2) a fragment of a polypeptide comprising the same domain. For example, an AP2 helix encoded by a SDF of the invention fused to second AP2 helix from ANT protein, which comprises two AP2 helices. The present invention also encompasses fusions of SDF encoded polypeptides, variants, or fragments thereof fused with related proteins or fragments thereof.

#### DEFINITION OF DOMAINS

The polypeptides of the invention may possess identifying domains as shown in TABLE 2. Specific domains within the SDF encoded polypeptides are indicated in TABLE 2. In addition, the domains within the SDF encoded polypeptide can be defined by the region that exhibits at least 70% sequence identity with the consensus sequences listed in the detailed in the protein domain table of each of the domains.

The majority of the protein domain descriptions given in the protein domain table are obtained from Prosite, (<http://www.expasy.ch/prosite/>), and Pfam,

(<http://pfam.wustl.edu/browse.shtml>).

A. Activities of Polypeptides Comprising Signal Peptides

Polypeptides comprising signal peptides are a family of proteins that are typically targeted to (1) a particular organelle or intracellular compartment, (2) interact with a particular molecule or (3) for secretion outside of a host cell. Example of polypeptides comprising signal peptides include, without limitation, secreted proteins, soluble proteins, receptors, proteins retained in the ER, etc.

These proteins comprising signal peptides are useful to modulate ligand-receptor interactions, cell-to-cell communication, signal transduction, intracellular communication, and activities and/or chemical cascades that take part in an organism outside or within of any particular cell.

One class of such proteins are soluble proteins which are transported out of the cell. These proteins can act as ligands that bind to receptor to trigger signal transduction or to permit communication between cells.

Another class is receptor proteins which also comprise a retention domain that lodges the receptor protein in the membrane when the cell transports the receptor to the surface of the cell. Like the soluble ligands, receptors can also modulate signal transduction and communication between cells.

In addition the signal peptide itself can serve as a ligand for some receptors. An example is the interaction of the ER targeting signal peptide with the signal recognition particle (SRP). Here, the SRP binds to the signal peptide, halting translation, and the resulting SRP complex then binds to docking proteins located on the surface of the ER, prompting transfer of the protein into the ER.

A description of signal peptide residue composition is described below in Subsection IV.C.1.

### III. Methods of Modulating Polypeptide Production

It is contemplated that polynucleotides of the invention can be incorporated into a host cell or in-vitro system to modulate polypeptide production. For instance, the SDFs prepared as described herein can be used to prepare expression cassettes useful in a number of techniques for suppressing or enhancing expression.

An example are polynucleotides comprising sequences to be transcribed, such as coding sequences, of the present invention can be inserted into nucleic acid constructs to modulate polypeptide production. Typically, such sequences to be transcribed are heterologous to at least one element of the nucleic acid construct to generate a chimeric gene or construct.

Another example of useful polynucleotides are nucleic acid molecules comprising regulatory sequences of the present invention. Chimeric genes or constructs can be generated when the regulatory sequences of the invention linked to heterologous sequences in a vector construct. Within the scope of invention are such chimeric gene and/or constructs.

Also within the scope of the invention are nucleic acid molecules, whereof at least a part or fragment of these DNA molecules are presented in Table 1 and Table 2, if present of the present application, and wherein the coding sequence is under the control of its own promoter and/or its own regulatory elements. Such molecules are useful for transforming the genome of a host cell or an organism regenerated from said host cell for modulating polypeptide production.

Additionally, a vector capable of producing the oligonucleotide can be inserted into the host cell to deliver the oligonucleotide.

More detailed description of components to be included in vector constructs are described both above and below.

Whether the chimeric vectors or native nucleic acids are utilized, such polynucleotides can be incorporated into a host cell to modulate polypeptide production. Native genes and/or nucleic acid molecules can be effective when exogenous to the host cell.

Methods of modulating polypeptide expression includes, without limitation:

Suppression methods, such as

Antisense

Ribozymes

Co-suppression

Insertion of Sequences into the Gene to be Modulated  
Regulatory Sequence Modulation.

as well as Methods for Enhancing Production, such as

5                   Insertion of Exogenous Sequences; and  
                  Regulatory Sequence Modulation.

**III.A. Suppression**

Expression cassettes of the invention can be used to suppress expression of endogenous genes which comprise the SDF sequence. Inhibiting expression can be useful, 10 for instance, to tailor the ripening characteristics of a fruit (Oeller et al., *Science* 254:437 (1991)) or to influence seed size (WO98/07842) or to provoke cell ablation (Mariani et al., *Nature* 357: 384-387 (1992)).

As described above, a number of methods can be used to inhibit gene expression in plants, such as antisense, ribozyme, introduction of exogenous genes into a host cell, 15 insertion of a polynucleotide sequence into the coding sequence and/or the promoter of the endogenous gene of interest, and the like.

**III.A.1. Antisense**

An expression cassette as described above can be transformed into host cell or plant to produce an antisense strand of RNA. For plant cells, antisense RNA inhibits gene 20 expression by preventing the accumulation of mRNA which encodes the enzyme of interest, *see, e.g.*, Sheehy et al., *Proc. Nat. Acad. Sci. USA*, 85:8805 (1988), and Hiatt et al., U.S. Patent No. 4,801,340.

**III.A.2. Ribozymes**

Similarly, ribozyme constructs can be transformed into a plant to cleave mRNA 25 and down-regulate translation.

**III.A.3. Co-Suppression**

Another method of suppression is by introducing an exogenous copy of the gene to be suppressed. Introduction of expression cassettes in which a nucleic acid is 30 configured in the sense orientation with respect to the promoter has been shown to prevent the accumulation of mRNA. A detailed description of this method is described above.

III.A.4. Insertion of Sequences into the Gene to be Modulated

Yet another means of suppressing gene expression is to insert a polynucleotide into the gene of interest to disrupt transcription or translation of the gene.

Homologous recombination could be used to target a polynucleotide insert to a gene using the Cre-Lox system (A.C. Vergunst et al., *Nucleic Acids Res.* 26:2729 (1998), A.C. Vergunst et al., *Plant Mol. Biol.* 38:393 (1998), H. Albert et al., *Plant J.* 7:649 (1995)).

In addition, random insertion of polynucleotides into a host cell genome can also be used to disrupt the gene of interest. Azpiroz-Leehan et al., *Trends in Genetics* 13:152 (1997). In this method, screening for clones from a library containing random insertions is preferred for identifying those that have polynucleotides inserted into the gene of interest. Such screening can be performed using probes and/or primers described above based on sequences from Table 1 and Table 2, if present, fragments thereof, and substantially similar sequence thereto. The screening can also be performed by selecting clones or any transgenic plants having a desired phenotype.

15 III.A.5. Regulatory Sequence Modulation

The SDFs described in Table 1 and Table 2, if present, and fragments thereof are examples of nucleotides of the invention that contain regulatory sequences that can be used to suppress or inactivate transcription and/or translation from a gene of interest as discussed in I.C.5.

20

III.A.6. Genes Comprising Dominant-Negative Mutations

When suppression of production of the endogenous, native protein is desired it is often helpful to express a gene comprising a dominant negative mutation. Production of protein variants produced from genes comprising dominant negative mutations is a useful tool for research. Genes comprising dominant negative mutations can produce a variant polypeptide which is capable of competing with the native polypeptide, but which does not produce the native result. Consequently, over expression of genes comprising these mutations can titrate out an undesired activity of the native protein. For example, The product from a gene comprising a dominant negative mutation of a receptor can be used to constitutively activate or suppress a signal transduction cascade, allowing examination of the phenotype and thus the trait(s) controlled by that receptor and pathway. Alternatively, the protein

arising from the gene comprising a dominant-negative mutation can be an inactive enzyme still capable of binding to the same substrate as the native protein and therefore competes with such native protein.

Products from genes comprising dominant-negative mutations can also act

5 upon the native protein itself to prevent activity. For example, the native protein may be active only as a homo-multimer or as one subunit of a hetero-multimer. Incorporation of an inactive subunit into the multimer with native subunit(s) can inhibit activity.

Thus, gene function can be modulated in host cells of interest by insertion into these cells vector constructs comprising a gene comprising a dominant-negative mutation.

10 **III.B. Enhanced Expression**

Enhanced expression of a gene of interest in a host cell can be accomplished by either (1) insertion of an exogenous gene; or (2) promoter modulation.

**III.B.1. Insertion of an Exogenous Gene**

15 Insertion of an expression construct encoding an exogenous gene can boost the number of gene copies expressed in a host cell.

Such expression constructs can comprise genes that either encode the native protein that is of interest or that encode a variant that exhibits enhanced activity as compared to the native protein. Such genes encoding proteins of interest can be constructed from the sequences from Table 1 and Table 2, if present, fragments thereof, and substantially similar sequence thereto.

Such an exogenous gene can include either a constitutive promoter permitting expression in any cell in a host organism or a promoter that directs transcription only in particular cells or times during a host cell life cycle or in response to environmental stimuli.

**III.B.2. Regulatory Sequence Modulation**

25 The SDFs of Table 1 and Table 2, if present, and fragments thereof, contain regulatory sequences that can be used to enhance expression of a gene of interest. For example, some of these sequences contain useful enhancer elements. In some cases, duplication of enhancer elements or insertion of exogenous enhancer elements will increase expression of a desired gene from a particular promoter. As other examples, all 11 promoters 30 require binding of a regulatory protein to be activated, while some promoters may need a protein that signals a promoter binding protein to expose a polymerase binding site. In either

case, over-production of such proteins can be used to enhance expression of a gene of interest by increasing the activation time of the promoter.

Such regulatory proteins are encoded by some of the sequences in Table 1 and Table 2, if present, fragments thereof, and substantially similar sequences thereto.

5

Coding sequences for these proteins can be constructed as described above.

#### IV. Gene Constructs and Vector Construction

To use isolated SDFs of the present invention or a combination of them or parts and/or mutants and/or fusions of said SDFs in the above techniques, recombinant DNA vectors which comprise said SDFs and are suitable for transformation of cells, such as plant cells, are usually prepared. The SDF construct can be made using standard recombinant DNA techniques (Sambrook et al. 1989) and can be introduced to the species of interest by *Agrobacterium*-mediated transformation or by other means of transformation (e.g., particle gun bombardment) as referenced below.

15 The vector backbone can be any of those typical in the art such as plasmids, viruses, artificial chromosomes, BACs, YACs and PACs and vectors of the sort described by

- (a) **BAC:** Shizuya et al., Proc. Natl. Acad. Sci. USA 89: 8794-8797 (1992);  
Hamilton et al., Proc. Natl. Acad. Sci. USA 93: 9975-9979 (1996);
- (b) **YAC:** Burke et al., Science 236:806-812 (1987);
- 20 (c) **PAC:** Sternberg N. et al., Proc Natl Acad Sci U S A. Jan;87(1):103-7 (1990);
- (d) **Bacteria-Yeast Shuttle Vectors:** Bradshaw et al., Nucl Acids Res 23: 4850-4856 (1995);
- (e) **Lambda Phage Vectors:** Replacement Vector, e.g.,  
Frischauf et al., J. Mol Biol 170: 827-842 (1983); or Insertion vector, e.g.,  
25 Huynh et al., In: Glover NM (ed) DNA Cloning: A practical Approach, Vol.1 Oxford: IRL Press (1985);
- (f) **T-DNA gene fusion vectors :** Walden et al., Mol Cell Biol 1: 175-194 (1990); and
- 30 (g) **Plasmid vectors:** Sambrook et al., *infra*.

Typically, a vector will comprise the exogenous gene, which in its turn comprises an SDF of the present invention to be introduced into the genome of a host cell, and which gene may be an antisense construct, a ribozyme construct chimeraplant, or a coding sequence with

any desired transcriptional and/or translational regulatory sequences, such as promoters, UTRs, and 3' end termination sequences. Vectors of the invention can also include origins of replication, scaffold attachment regions (SARs), markers, homologous sequences, introns, etc.

A DNA sequence coding for the desired polypeptide, for example a cDNA sequence encoding a full length protein, will preferably be combined with transcriptional and translational initiation regulatory sequences which will direct the transcription of the sequence from the gene in the intended tissues of the transformed plant.

For example, for over-expression, a plant promoter fragment may be employed that will direct transcription of the gene in all tissues of a regenerated plant. Alternatively, the plant promoter may direct transcription of an SDF of the invention in a specific tissue (tissue-specific promoters) or may be otherwise under more precise environmental control (inducible promoters).

If proper polypeptide production is desired, a polyadenylation region at the 3'-end of the coding region is typically included. The polyadenylation region can be derived from the natural gene, from a variety of other plant genes, or from T-DNA.

The vector comprising the sequences from genes or SDF or the invention may comprise a marker gene that confers a selectable phenotype on plant cells. The vector can include promoter and coding sequence, for instance. For example, the marker may encode biocide resistance, particularly antibiotic resistance, such as resistance to kanamycin, G418, bleomycin, hygromycin, or herbicide resistance, such as resistance to chlorosulfuron or phosphinotricin.

#### IV.A. Coding Sequences

Generally, the sequence in the transformation vector and to be introduced into the genome of the host cell does not need to be absolutely identical to an SDF of the present invention. Also, it is not necessary for it to be full length, relative to either the primary transcription product or fully processed mRNA. Furthermore, the introduced sequence need not have the same intron or exon pattern as a native gene. Also, heterologous non-coding segments can be incorporated into the coding sequence without changing the desired amino acid sequence of the polypeptide to be produced.

#### IV.B. Promoters

As explained above, introducing an exogenous SDF from the same species or an orthologous SDF from another species can modulate the expression of a native gene

corresponding to that SDF of interest. Such an SDF construct can be under the control of either a constitutive promoter or a highly regulated inducible promoter (e.g., a copper inducible promoter). The promoter of interest can initially be either endogenous or heterologous to the species in question. When re-introduced into the genome of said species, 5 such promoter becomes exogenous to said species. Over-expression of an SDF transgene can lead to co-suppression of the homologous endogenous sequence thereby creating some alterations in the phenotypes of the transformed species as demonstrated by similar analysis of the chalcone synthase gene (Napoli et al., *Plant Cell* 2:279 (1990) and van der Krol et al., *Plant Cell* 2:291 (1990)). If an SDF is found to encode a protein with desirable 10 characteristics, its over-production can be controlled so that its accumulation can be manipulated in an organ- or tissue-specific manner utilizing a promoter having such specificity.

Likewise, if the promoter of an SDF (or an SDF that includes a promoter) is found to be tissue-specific or developmentally regulated, such a promoter can be utilized to drive 15 or facilitate the transcription of a specific gene of interest (e.g., seed storage protein or root-specific protein). Thus, the level of accumulation of a particular protein can be manipulated or its spatial localization in an organ- or tissue-specific manner can be altered.

#### IV. C Signal Peptides

20 SDFs of the present invention containing signal peptides are indicated in Table 1 and Table 2, if present. In some cases it may be desirable for the protein encoded by an introduced exogenous or orthologous SDF to be targeted (1) to a particular organelle intracellular compartment, (2) to interact with a particular molecule such as a membrane molecule or (3) for secretion outside of the cell harboring the introduced SDF. This will be 25 accomplished using a signal peptide.

Signal peptides direct protein targeting, are involved in ligand-receptor interactions and act in cell to cell communication. Many proteins, especially soluble proteins, contain a signal peptide that targets the protein to one of several different intracellular compartments. In plants, these compartments include, but are not limited to, the endoplasmic reticulum 30 (ER), mitochondria, plastids (such as chloroplasts), the vacuole, the Golgi apparatus, protein storage vesicles (PSV) and, in general, membranes. Some signal peptide sequences are conserved, such as the Asn-Pro-Ile-Arg amino acid motif found in the N-terminal propeptide signal that targets proteins to the vacuole (Marty (1999) *The Plant Cell* 11: 587-599). Other signal peptides do not have a consensus sequence *per se*, but are largely

composed of hydrophobic amino acids, such as those signal peptides targeting proteins to the ER (Vitale and Denecke (1999) *The Plant Cell* 11: 615-628). Still others do not appear to contain either a consensus sequence or an identified common secondary sequence, for instance the chloroplast stromal targeting signal peptides (Keegstra and Cline (1999) *The Plant Cell* 11: 557-570). Furthermore, some targeting peptides are bipartite, directing proteins first to an organelle and then to a membrane within the organelle (e.g. within the thylakoid lumen of the chloroplast; see Keegstra and Cline (1999) *The Plant Cell* 11: 557-570). In addition to the diversity in sequence and secondary structure, placement of the signal peptide is also varied. Proteins destined for the vacuole, for example, have targeting signal peptides found at the N-terminus, at the C-terminus and at a surface location in mature, folded proteins. Signal peptides also serve as ligands for some receptors.

These characteristics of signal proteins can be used to more tightly control the phenotypic expression of introduced SDFs. In particular, associating the appropriate signal sequence with a specific SDF can allow sequestering of the protein in specific organelles (plastids, as an example), secretion outside of the cell, targeting interaction with particular receptors, etc. Hence, the inclusion of signal proteins in constructs involving the SDFs of the invention increases the range of manipulation of SDF phenotypic expression. The nucleotide sequence of the signal peptide can be isolated from characterized genes using common molecular biological techniques or can be synthesized *in vitro*.

In addition, the native signal peptide sequences, both amino acid and nucleotide, described in Table 1 and Table 2, if present can be used to modulate polypeptide transport. Further variants of the native signal peptides described in Table 1 and Table 2, if present are contemplated. Insertions, deletions, or substitutions can be made. Such variants will retain at least one of the functions of the native signal peptide as well as exhibiting some degree of sequence identity to the native sequence.

Also, fragments of the signal peptides of the invention are useful and can be fused with other signal peptides of interest to modulate transport of a polypeptide.

## 20 V. Transformation Techniques

A wide range of techniques for inserting exogenous polynucleotides are known for a number of host cells, including, without limitation, bacterial, yeast, mammalian, insect and plant cells.

Techniques for transforming a wide variety of higher plant species are well known and described in the technical and scientific literature. See, e.g. Weising et al., *Ann. Rev. Genet.* 22:421 (1988); and Christou, *Euphytica*, v. 85, n.1-3:13-27, (1995).

DNA constructs of the invention may be introduced into the genome of the desired plant host by a variety of conventional techniques. For example, the DNA construct may be introduced directly into the genomic DNA of the plant cell using techniques such as electroporation and microinjection of plant cell protoplasts, or the DNA constructs can be introduced directly to plant tissue using ballistic methods, such as DNA particle bombardment. Alternatively, the DNA constructs may be combined with suitable T-DNA flanking regions and introduced into a conventional *Agrobacterium tumefaciens* host vector. The virulence functions of the *Agrobacterium tumefaciens* host will direct the insertion of the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria (McCormac et al., *Mol. Biotechnol.* 8:199 (1997); Hamilton, *Gene* 200:107 (1997)); Salomon et al. *EMBO J.* 3:141 (1984); Herrera-Estrella et al. *EMBO J.* 2:987 (1983).

Microinjection techniques are known in the art and well described in the scientific and patent literature. The introduction of DNA constructs using polyethylene glycol precipitation is described in Paszkowski et al. *EMBO J.* 3:2717 (1984). Electroporation techniques are described in Fromm et al. *Proc. Natl. Acad. Sci. USA* 82:5824 (1985). Ballistic transformation techniques are described in Klein et al. *Nature* 327:773 (1987). *Agrobacterium tumefaciens*-mediated transformation techniques, including disarming and use of binary or co-integrate vectors, are well described in the scientific literature. See, for example Hamilton, *CM., Gene* 200:107 (1997); Müller et al. *Mol. Gen. Genet.* 207:171 (1987); Komari et al. *Plant J.* 10:165 (1996); Venkateswarlu et al. *Biotechnology* 9:1103 (1991) and Gleave, *AP., Plant Mol. Biol.* 20:1203 (1992); Graves and Goldman, *Plant Mol. Biol.* 7:34 (1986) and Gould et al., *Plant Physiology* 95:426 (1991).

Transformed plant cells which are derived by any of the above transformation techniques can be cultured to regenerate a whole plant that possesses the transformed genotype and thus the desired phenotype such as seedlessness. Such regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker which has been introduced together with the desired nucleotide sequences. Plant regeneration from cultured protoplasts is described in Evans et al., *Protoplasts Isolation and Culture* in "Handbook of Plant Cell Culture," pp. 124-176, MacMillan Publishing Company, New York, 1983; and Binding, *Regeneration of Plants, Plant Protoplasts*, pp. 21-73, CRC Press, Boca Raton, 1988. Regeneration can also be

obtained from plant callus, explants, organs, or parts thereof. Such regeneration techniques are described generally in Klee et al. *Ann. Rev. of Plant Phys.* 38:467 (1987). Regeneration of monocots (rice) is described by Hosoyama et al. (*Biosci. Biotechnol. Biochem.* 58:1500 (1994)) and by Ghosh et al. (*J. Biotechnol.* 32:1 (1994)). The nucleic acids of the invention can 5 be used to confer desired traits on essentially any plant.

Thus, the invention has use over a broad range of plants, including species from the genera *Anacardium*, *Arachis*, *Asparagus*, *Atropa*, *Avena*, *Brassica*, *Citrus*, *Citrullus*, *Capsicum*, *Carthamus*, *Cocos*, *Coffea*, *Cucumis*, *Cucurbita*, *Daucus*, *Elaeis*, *Fragaria*, *Glycine*, *Gossypium*, *Helianthus*, *Heterocallis*, *Hordeum*, *Hyoscyamus*, *Lactuca*, *Linum*, 10 *Lolium*, *Lupinus*, *Lycopersicon*, *Malus*, *Manihot*, *Majorana*, *Medicago*, *Nicotiana*, *Olea*, *Oryza*, *Panicum*, *Pannisetum*, *Persea*, *Phaseolus*, *Pistacia*, *Pisum*, *Pyrus*, *Prunus*, *Raphanus*, *Ricinus*, *Secale*, *Senecio*, *Sinapis*, *Solanum*, *Sorghum*, *Theobromus*, *Trigonella*, *Triticum*, *Vicia*, *Vitis*, *Vigna*, and, *Zea*.

One of skill will recognize that after the expression cassette is stably incorporated in 15 transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

The particular sequences of SDFs identified are provided in the attached Table 1 and 20 Table 2, if present. One of ordinary skill in the art, having this data, can obtain cloned DNA fragments, synthetic DNA fragments or polypeptides constituting desired sequences by recombinant methodology known in the art or described herein.

## EXAMPLES

The invention is illustrated by way of the following examples. The invention is not limited by these examples as the scope of the invention is defined solely by the claims 25 following.

### EXAMPLE 1: cDNA PREPARATION

A number of the nucleotide sequences disclosed in Table 1 and Table 2, if present herein as representative of the SDFs of the invention can be obtained by sequencing genomic DNA (gDNA) and/or cDNA from corn plants grown from HYBRID SEED # 35A19, 30 purchased from Pioneer Hi-Bred International, Inc., Supply Management, P.O. Box 256, Johnston, Iowa 50131-0256.

A number of the nucleotide sequences disclosed in Table 1 and Table 2, if present herein as representative of the SDFs of the invention can also be obtained by sequencing genomic DNA from *Arabidopsis thaliana*, Wassilewskija ecotype or by sequencing cDNA obtained from mRNA from such plants as described below. This is a true breeding strain.

5 Seeds of the plant are available from the Arabidopsis Biological Resource Center at the Ohio State University, under the accession number CS2360. Seeds of this plant were deposited under the terms and conditions of the Budapest Treaty at the American Type Culture Collection, Manassas, VA on August 31, 1999, and were assigned ATCC No. PTA-595.

10 Other methods for cloning full-length cDNA are described, for example, by Seki et al., *Plant Journal* 15:707-720 (1998) "High-efficiency cloning of Arabidopsis full-length cDNA by biotinylated Cap trapper"; Maruyama et al., *Gene* 138:171 (1994) "Oligo-capping a simple method to replace the cap structure of eukaryotic mRNAs with oligoribonucleotides"; and WO 96/34981.

15 Tissues were, or each organ was, individually pulverized and frozen in liquid nitrogen. Next, the samples were homogenized in the presence of detergents and then centrifuged. The debris and nuclei were removed from the sample and more detergents were added to the sample. The sample was centrifuged and the debris was removed. Then the sample was applied to a 2M sucrose cushion to isolate polysomes. The RNA was 20 isolated by treatment with detergents and proteinase K followed by ethanol precipitation and centrifugation. The polysomal RNA from the different tissues was pooled according to the following mass ratios: 15/15/1 for male inflorescences, female inflorescences and root, respectively. The pooled material was then used for cDNA synthesis by the methods described below.

25 Starting material for cDNA synthesis for the exemplary corn cDNA clones with sequences presented in Table 1 and Table 2, if present was poly(A)-containing polysomal mRNAs from inflorescences and root tissues of corn plants grown from HYBRID SEED # 35A19. Male inflorescences and female (pre-and post-fertilization) inflorescences were isolated at various stages of development. Selection for poly(A) 30 containing polysomal RNA was done using oligo d(T) cellulose columns, as described by Cox and Goldberg, "Plant Molecular Biology: A Practical Approach", pp. 1-35, Shaw ed., c. 1988 by IRL, Oxford. The quality and the integrity of the polyA+ RNAs were evaluated.

Starting material for cDNA synthesis for the exemplary *Arabidopsis* cDNA clones with sequences presented in Table 1 and Table 2, if present was polysomal RNA isolated from the top-most inflorescence tissues of *Arabidopsis thaliana* Wassilewskija (Ws.) and from roots of *Arabidopsis thaliana* Landsberg erecta (L. er.), also obtained from the Arabidopsis Biological Resource Center. Nine parts inflorescence to every part root was used, as measured by wet mass. Tissue was pulverized and exposed to liquid nitrogen. Next, the sample was homogenized in the presence of detergents and then centrifuged. The debris and nuclei were removed from the sample and more detergents were added to the sample. The sample was centrifuged and the debris was removed and the sample was applied to a 2M sucrose cushion to isolate polysomal RNA. Cox et al., "Plant Molecular Biology: A Practical Approach", pp. 1-35, Shaw ed., c. 1988 by IRL, Oxford. The polysomal RNA was used for cDNA synthesis by the methods described below. Polysomal mRNA was then isolated as described above for corn cDNA. The quality of the RNA was assessed electrophoretically.

Following preparation of the mRNAs from various tissues as described above, selection of mRNA with intact 5' ends and specific attachment of an oligonucleotide tag to the 5' end of such mRNA was performed using either a chemical or enzymatic approach. Both techniques take advantage of the presence of the "cap" structure, which characterizes the 5' end of most intact mRNAs and which comprises a guanosine generally methylated once, at the 7 position.

The chemical modification approach involves the optional elimination of the 2', 3'-cis diol of the 3' terminal ribose, the oxidation of the 2', 3'-cis diol of the ribose linked to the cap of the 5' ends of the mRNAs into a dialdehyde, and the coupling of the such obtained dialdehyde to a derivatized oligonucleotide tag. Further detail regarding the chemical approaches for obtaining mRNAs having intact 5' ends are disclosed in International Application No. WO96/34981 published November 7, 1996.

The enzymatic approach for ligating the oligonucleotide tag to the intact 5' ends of mRNAs involves the removal of the phosphate groups present on the 5' ends of uncapped incomplete mRNAs, the subsequent decapping of mRNAs having intact 5' ends and the ligation of the phosphate present at the 5' end of the decapped mRNA to an oligonucleotide tag. Further detail regarding the enzymatic approaches for obtaining mRNAs having intact 5' ends are disclosed in Dumas Milne Edwards J.B. (Doctoral Thesis of Paris VI University, Le clonage des ADNc complets: difficultés et perspectives nouvelles. Apports pour l'étude de la

régulation de l'expression de la tryptophane hydroxylase de rat, 20 Dec. 1993), EP0 625572 and Kato *et al.*, *Gene* 150:243-250 (1994).

In both the chemical and the enzymatic approach, the oligonucleotide tag has a restriction enzyme site (e.g. an EcoRI site) therein to facilitate later cloning procedures.

5 Following attachment of the oligonucleotide tag to the mRNA, the integrity of the mRNA is examined by performing a Northern blot using a probe complementary to the oligonucleotide tag.

For the mRNAs joined to oligonucleotide tags using either the chemical or the enzymatic method, first strand cDNA synthesis is performed using an oligo-dT primer with 10 reverse transcriptase. This oligo-dT primer can contain an internal tag of at least 4 nucleotides, which can be different from one mRNA preparation to another. Methylated dCTP is used for cDNA first strand synthesis to protect the internal EcoRI sites from digestion during subsequent steps. The first strand cDNA is precipitated using isopropanol after removal of RNA by alkaline hydrolysis to eliminate residual primers.

15 Second strand cDNA synthesis is conducted using a DNA polymerase, such as Klenow fragment and a primer corresponding to the 5' end of the ligated oligonucleotide. The primer is typically 20-25 bases in length. Methylated dCTP is used for second strand synthesis in order to protect internal EcoRI sites in the cDNA from digestion during the cloning process.

20 Following second strand synthesis, the full-length cDNAs are cloned into a phagemid vector, such as pBlueScript<sup>TM</sup> (Stratagene). The ends of the full-length cDNAs are blunted with T4 DNA polymerase (Biolabs) and the cDNA is digested with EcoRI. Since methylated dCTP is used during cDNA synthesis, the EcoRI site present in the tag is the only hemi-methylated site; hence the only site susceptible to EcoRI digestion. In some instances, to facilitate subcloning, an Hind III adapter is added to the 3' end of full-length cDNAs.

25 The full-length cDNAs are then size fractionated using either exclusion chromatography (AcA, Biosepra) or electrophoretic separation which yields 3 to 6 different fractions. The full-length cDNAs are then directionally cloned either into pBlueScript<sup>TM</sup> using either the EcoRI and SmaI restriction sites or, when the Hind III adapter is present in the full-length cDNAs, the EcoRI and Hind III restriction sites. The ligation mixture is transformed, 30 preferably by electroporation, into bacteria, which are then propagated under appropriate antibiotic selection.

Clones containing the oligonucleotide tag attached to full-length cDNAs are selected as follows.

The plasmid cDNA libraries made as described above are purified (e.g. by a column available from Qiagen). A positive selection of the tagged clones is performed as follows. Briefly, in this selection procedure, the plasmid DNA is converted to single stranded DNA using phage F1 gene II endonuclease in combination with an exonuclease (Chang et al., *Gene* 127:95 (1993)) such as exonuclease III or T7 gene 6 exonuclease. The resulting single stranded DNA is then purified using paramagnetic beads as described by Fry et al., *Biotechniques* 13: 124 (1992). Here the single stranded DNA is hybridized with a biotinylated oligonucleotide having a sequence corresponding to the 3' end of the oligonucleotide tag. Preferably, the primer has a length of 20-25 bases. Clones including a sequence complementary to the biotinylated oligonucleotide are selected by incubation with streptavidin coated magnetic beads followed by magnetic capture. After capture of the positive clones, the plasmid DNA is released from the magnetic beads and converted into double stranded DNA using a DNA polymerase such as ThermoSequenase™ (obtained from Amersham Pharmacia Biotech). Alternatively, protocols such as the Gene Trapper™ kit (Gibco BRL) can be used. The double stranded DNA is then transformed, preferably by electroporation, into bacteria. The percentage of positive clones having the 5' tag oligonucleotide is typically estimated to be between 90 and 98% from dot blot analysis.

Following transformation, the libraries are ordered in microtiter plates and sequenced. The *Arabidopsis* library was deposited at the American Type Culture Collection on January 7, 2000 as "E-coli liba 010600" under the accession number **PTA-1161**.

#### **EXAMPLE 2: SOUTHERN HYBRIDIZATIONS**

The SDFs of the invention can be used in Southern hybridizations as described above. The following describes extraction of DNA from nuclei of plant cells, digestion of the nuclear DNA and separation by length, transfer of the separated fragments to membranes, preparation of probes for hybridization, hybridization and detection of the hybridized probe.

The procedures described herein can be used to isolate related polynucleotides or for diagnostic purposes. Moderate stringency hybridization conditions, as defined above, are described in the present example. These conditions result in detection of hybridization between sequences having at least 70% sequence identity. As described above, the hybridization and wash conditions can be changed to reflect the desired percentage of sequence identity between probe and target sequences that can be detected.

In the following procedure, a probe for hybridization is produced from two PCR reactions using two primers from genomic sequence of *Arabidopsis thaliana*. As described above, the particular template for generating the probe can be any desired template.

5 The first PCR product is assessed to validate the size of the primer to assure it is of the expected size. Then the product of the first PCR is used as a template, with the same pair of primers used in the first PCR, in a second PCR that produces a labeled product used as the probe.

10 Fragments detected by hybridization, or other bands of interest, can be isolated from gels used to separate genomic DNA fragments by known methods for further purification and/or characterization.

### Buffers for nuclear DNA extraction

#### 1. 10X HB

	1000 ml	
40 mM spermidine	10.2 g	Spermine (Sigma S-2876) and spermidine (Sigma S-2501)
10 mM spermine	3.5 g	Stabilize chromatin and the nuclear membrane
0.1 M EDTA (disodium)	37.2 g	EDTA inhibits nuclease
0.1 M Tris	12.1 g	Buffer
0.8 M KCl	59.6 g	Adjusts ionic strength for stability of nuclei

Adjust pH to 9.5 with 10 N NaOH. It appears that there is a nuclease present in leaves. Use of pH 9.5 appears to inactivate this nuclease.

15 2. 2 M sucrose (684 g per 1000 ml)

Heat about half the final volume of water to about 50°C. Add the sucrose slowly then bring the mixture to close to final volume; stir constantly until it has dissolved. Bring the solution to volume.

## 3. Sarkosyl solution (lyses nuclear membranes)

		<u>1000 ml</u>
	N-lauroyl sarcosine (Sarkosyl)	20.0 g
	0.1 M Tris	12.1 g
5	0.04 M EDTA (Disodium)	14.9 g

Adjust the pH to 9.5 after all the components are dissolved and bring up to the proper volume.

## 4. 20% Triton X-100

80 ml Triton X-100

10 320 ml 1xHB (w/o  $\beta$ -ME and PMSF)

Prepare in advance; Triton takes some time to dissolve

A. Procedure

## 1. Prepare 1X "H" buffer (keep ice-cold during use)

		<u>1000 ml</u>
15	10X HB	100 ml
	2 M sucrose	250 ml a non-ionic osmoticum
	Water	634 ml

**Added just before use:**

100 mM PMSF\* 10 ml a protease inhibitor; protects nuclear membrane proteins

20  $\beta$ -mercaptoethanol 1 ml inactivates nuclease by reducing disulfide bonds

\*100 mM PMSF

(phenyl methyl sulfonyl fluoride, Sigma P-7626)

25 (add 0.0875 g to 5 ml 100% ethanol)

2. Homogenize the tissue in a blender (use 300-400 ml of 1xHB per blender). Be sure that you use 5-10 ml of HB buffer per gram of tissue. Blenders generate heat so be sure to keep the homogenate cold. It is necessary to put the blenders in ice periodically.

5 3. Add the 20% Triton X-100 (25 ml per liter of homogenate) and gently stir on ice for 20 min. This lyses plastid, but not nuclear, membranes.

4. Filter the tissue suspension through several nylon filters into an ice-cold beaker. The first filtration is through a 250-micron membrane; the second is through an 85-micron membrane; the third is through a 50-micron membrane; and the fourth is through a 20-micron membrane. Use a large funnel to hold the filters. Filtration can be sped up by gently squeezing the liquid through the filters.

10 5. Centrifuge the filtrate at 1200 x g for 20 min. at 4°C to pellet the nuclei.

6. Discard the dark green supernatant. The pellet will have several layers to it. One is starch; it is white and gritty. The nuclei are gray and soft. In the early steps, there 15 may be a dark green and somewhat viscous layer of chloroplasts.

Wash the pellets in about 25 ml cold H buffer (with Triton X-100) and resuspend by swirling gently and pipetting. After the pellets are resuspended.

Pellet the nuclei again at 1200 - 1300 x g. Discard the supernatant.

20 Repeat the wash 3-4 times until the supernatant has changed from a dark green to a pale green. This usually happens after 3 or 4 resuspensions. At this point, the pellet is typically grayish white and very slippery. The Triton X-100 in these repeated steps helps to destroy the chloroplasts and mitochondria that contaminate the prep.

Resuspend the nuclei for a final time in a total of 15 ml of H buffer and transfer the suspension to a sterile 125 ml Erlenmeyer flask.

7. Add 15 ml, dropwise, cold 2% Sarkosyl, 0.1 M Tris, 0.04 M EDTA solution (pH 9.5) while swirling gently. This lyses the nuclei. The solution will become very viscous.
8. Add 30 grams of CsCl and gently swirl at room temperature until the CsCl is in solution. The mixture will be gray, white and viscous.  
5
9. Centrifuge the solution at 11,400 x g at 4°C for at least 30 min. The longer this spin is, the firmer the protein pellicle.
10. The result is typically a clear green supernatant over a white pellet, and (perhaps) under a protein pellicle. Carefully remove the solution under the protein pellicle and above the pellet. Determine the density of the solution by weighing 1 ml of solution and add CsCl if necessary to bring to 1.57 g/ml. The solution contains dissolved solids (sucrose etc) and the refractive index alone will not be an accurate guide to CsCl concentration.  
10
11. Add 20  $\mu$ l of 10 mg/ml EtBr per ml of solution.  
15
12. Centrifuge at 184,000 x g for 16 to 20 hours in a fixed-angle rotor.
13. Remove the dark red supernatant that is at the top of the tube with a plastic transfer pipette and discard. Carefully remove the DNA band with another transfer pipette. The DNA band is usually visible in room light; otherwise, use a long wave UV light  
20 to locate the band.
14. Extract the ethidium bromide with isopropanol saturated with water and salt. Once the solution is clear, extract at least two more times to ensure that all of the EtBr is gone. Be very gentle, as it is very easy to shear the DNA at this step. This extraction may take a while because the DNA solution tends to be very viscous. If  
25 the solution is too viscous, dilute it with TE.
15. Dialyze the DNA for at least two days against several changes (at least three times) of TE (10 mM Tris, 1mM EDTA, pH 8) to remove the cesium chloride.

16. Remove the dialyzed DNA from the tubing. If the dialyzed DNA solution contains a lot of debris, centrifuge the DNA solution at least at 2500 x g for 10 min. and carefully transfer the clear supernatant to a new tube. Read the A260 concentration of the DNA.
- 5 17. Assess the quality of the DNA by agarose gel electrophoresis (1% agarose gel) of the DNA. Load 50 ng and 100 ng (based on the OD reading) and compare it with known and good quality DNA. Undigested lambda DNA and a lambda-HindIII-digested DNA are good molecular weight makers.

#### **Protocol for Digestion of Genomic DNA**

##### **Protocol:**

- 10 1. The relative amounts of DNA for different crop plants that provide approximately a balanced number of genome equivalent is given in Table A. Note that due to the size of the wheat genome, wheat DNA will be underrepresented. Lambda DNA provides a useful control for complete digestion.
- 15 2. Precipitate the DNA by adding 3 volumes of 100% ethanol. Incubate at -20°C for at least two hours. Yeast DNA can be purchased and made up at the necessary concentration, therefore no precipitation is necessary for yeast DNA.
- 20 3. Centrifuge the solution at 11,400 x g for 20 min. Decant the ethanol carefully (be careful not to disturb the pellet). Be sure that the residual ethanol is completely removed either by vacuum desiccation or by carefully wiping the sides of the tubes with a clean tissue.
4. Resuspend the pellet in an appropriate volume of water. Be sure the pellet is fully resuspended before proceeding to the next step. This may take about 30 min.
- 25 5. Add the appropriate volume of 10X reaction buffer provided by the manufacturer of the restriction enzyme to the resuspended DNA followed by the appropriate volume of enzymes. Be sure to mix it properly by slowly swirling the tubes.

6. Set-up the lambda digestion-control for each DNA that you are digesting.

7. Incubate both the experimental and lambda digests overnight at 37°C. Spin down condensation in a microfuge before proceeding.

8. After digestion, add 2 µl of loading dye (typically 0.25% bromophenol blue, 0.25% xylene cyanol in 15% Ficoll or 30% glycerol) to the lambda-control digests and load in 1% TPE-agarose gel (TPE is 90 mM Tris-phosphate, 2 mM EDTA, pH 8). If the lambda DNA in the lambda control digests are completely digested, proceed with the precipitation of the genomic DNA in the digests.

9. Precipitate the digested DNA by adding 3 volumes of 100% ethanol and incubating in -20°C for at least 2 hours (preferably overnight).

EXCEPTION: *Arabidopsis* and yeast DNA are digested in an appropriate volume; they don't have to be precipitated.

10. Resuspend the DNA in an appropriate volume of TE (e.g., 22 µl x 50 blots = 1100 µl) and an appropriate volume of 10X loading dye (e.g., 2.4 µl x 50 blots = 120 µl). Be careful in pipetting the loading dye - it is viscous. Be sure you are pipetting the correct volume.

Table A  
Some guide points in digesting genomic DNA.

Species	Genome Size	Size Relative to <i>Arabidopsis</i>	Genome Equivalent to 2 µg <i>Arabidopsis</i> DNA	Amount of DNA per blot
<i>Arabidopsis</i>	120 Mb	1X	1X	2 µg
<i>Brassica</i>	1,100 Mb	9.2X	0.54X	10 µg
Corn	2,800 Mb	23.3X	0.43X	20 µg
Cotton	2,300 Mb	19.2X	0.52X	20 µg

Oat	11,300 Mb	94X	0.11X	20 µg
Rice	400 Mb	3.3X	0.75X	5 µg
Soybean	1,100 Mb	9.2X	0.54X	10 µg
Sugarbeet	758 Mb	6.3X	0.8X	10 µg
Sweetclover	1,100 Mb	9.2X	0.54X	10 µg
Wheat	16,000 Mb	133X	0.08X	20 µg
Yeast	15 Mb	0.12X	1X	0.25 µg

### Protocol for Southern Blot Analysis

The digested DNA samples are electrophoresed in 1% agarose gels in 1x TPE buffer. Low voltage; overnight separations are preferred. The gels are stained with EtBr and

5 photographed.

1. For blotting the gels, first incubate the gel in 0.25 N HCl (with gentle shaking) for about 15 min.

10 2. Then briefly rinse with water. The DNA is denatured by 2 incubations. Incubate (with shaking) in 0.5 M NaOH in 1.5 M NaCl for 15 min.

3. The gel is then briefly rinsed in water and neutralized by incubating twice (with shaking) in 1.5 M Tris pH 7.5 in 1.5 M NaCl for 15 min.

15 4. A nylon membrane is prepared by soaking it in water for at least 5 min, then in 6X SSC for at least 15 min. before use. (20x SSC is 175.3 g NaCl, 88.2 g sodium citrate per liter, adjusted to pH 7.0.)

20 5. The nylon membrane is placed on top of the gel and all bubbles in between are removed. The DNA is blotted from the gel to the membrane using an absorbent medium, such as paper toweling and 6x SCC buffer. After the transfer, the membrane may be lightly brushed with a gloved hand to remove any agarose sticking to the surface.

6. The DNA is then fixed to the membrane by UV crosslinking and baking at 80°C. The membrane is stored at 4°C until use.

B. Protocol for PCR Amplification of Genomic Fragments in Arabidopsis

Amplification procedures:

5 1. Mix the following in a 0.20 ml PCR tube or 96-well PCR plate:

Volume	Stock	Final Amount or Conc.
0.5 $\mu$ l	~ 10 ng/ $\mu$ l genomic DNA <sup>1</sup>	5 ng
2.5 $\mu$ l	<b>10X PCR buffer</b>	20 mM Tris, 50 mM KCl
0.75 $\mu$ l	50 mM MgCl <sub>2</sub>	1.5 mM
1 $\mu$ l	10 pmol/ $\mu$ l Primer 1 (Forward)	10 pmol
1 $\mu$ l	10 pmol/ $\mu$ l Primer 2 (Reverse)	10 pmol
0.5 $\mu$ l	5 mM dNTPs	0.1 mM
0.1 $\mu$ l	5 units/ $\mu$ l Platinum Taq <sup>TM</sup> (Life Technologies, Gaithersburg, MD) DNA Polymerase	1 units
(to 25 $\mu$ l)	<b>Water</b>	

2. The template DNA is amplified using a Perkin Elmer 9700 PCR machine:

1) 94°C for 10 min. followed by

<u>2)</u> 5 cycles:	<u>3)</u> 5 cycles:	<u>4)</u> 25 cycles:
94 °C - 30 sec	94 °C - 30 sec	94 °C - 30 sec
62 °C - 30 sec	58 °C - 30 sec	53 °C - 30 sec
72 °C - 3 min	72 °C - 3 min	72 °C - 3 min

5) 72 °C for 7 min. Then the reactions are stopped by chilling to 4 °C.

The procedure can be adapted to a multi-well format if necessary.

**Quantification and Dilution of PCR Products:**

5        1. The product of the PCR is analyzed by electrophoresis in a 1% agarose gel. A linearized plasmid DNA can be used as a quantification standard (usually at 50, 100, 200, and 400 ng). These will be used as references to approximate the amount of PCR products. HindIII-digested Lambda DNA is useful as a molecular weight marker. The gel can be run fairly quickly; e.g., at 100 volts. The standard gel is examined to determine that the size of the PCR products is consistent with the expected size and if there are significant extra bands or smearable products in the PCR reactions.

10        2. The amounts of PCR products can be estimated on the basis of the plasmid standard.

15        3. For the small number of reactions that produce extraneous bands, a small amount of DNA from bands with the correct size can be isolated by dipping a sterile 10-μl tip into the band while viewing through a UV Transilluminator. The small amount of agarose gel (with the DNA fragment) is used in the labeling reaction.

**C. Protocol for PCR-DIG-Labeling of DNA**

Solutions:

20        Reagents in PCR reactions (diluted PCR products, 10X PCR Buffer, 50 mM MgCl<sub>2</sub>, 5 U/μl Platinum Taq Polymerase, and the primers)

---

<sup>1</sup> *Arabidopsis* DNA is used in the present experiment, but the procedure is a general one.

10X dNTP + DIG-11-dUTP [1:5]: (2 mM dATP, 2 mM dCTP, 2 mM dGTP, 1.65 mM dTTP, 0.35 mM DIG-11-dUTP)

10X dNTP + DIG-11-dUTP [1:10]: (2 mM dATP, 2 mM dCTP, 2 mM dGTP, 1.81 mM dTTP, 0.19 mM DIG-11-dUTP)

5 10X dNTP + DIG-11-dUTP [1:15]: (2 mM dATP, 2 mM dCTP, 2 mM dGTP, 1.875 mM dTTP, 0.125 mM DIG-11-dUTP)

TE buffer (10 mM Tris, 1 mM EDTA, pH 8)

10 Maleate buffer: In 700 ml of deionized distilled water, dissolve 11.61 g maleic acid and 8.77 g NaCl. Add NaOH to adjust the pH to 7.5. Bring the volume to 1 L. Stir for 15 min. and sterilize.

15 10% blocking solution: In 80 ml deionized distilled water, dissolve 1.16g maleic acid. Next, add NaOH to adjust the pH to 7.5. Add 10 g of the blocking reagent powder (Boehringer Mannheim, Indianapolis, IN, Cat. no. 1096176). Heat to 60°C while stirring to dissolve the powder. Adjust the volume to 100 ml with water. Stir and sterilize.

1% blocking solution: Dilute the 10% stock to 1% using the maleate buffer.

Buffer 3 (100 mM Tris, 100 mM NaCl, 50 mM MgCl<sub>2</sub>, pH9.5). Prepared from autoclaved solutions of 1M Tris pH 9.5, 5 M NaCl, and 1 M MgCl<sub>2</sub> in autoclaved distilled water.

Procedure:

1. PCR reactions are performed in 25  $\mu$ l volumes containing:

PCR buffer	1X
MgCl <sub>2</sub>	1.5 mM
5 10X dNTP + DIG-11-dUTP	1X (please see the note below)
Platinum Taq™ Polymerase	1 unit
10 pg probe DNA	
10 pmol primer 1	

**Note:** Use for:

10	<u>10X dNTP + DIG-11-dUTP (1:5)</u>	<u>&lt; 1 kb</u>
	10X dNTP + DIG-11-dUTP (1:10)	1 kb to 1.8 kb
	10X dNTP + DIG-11-dUTP (1:15)	> 1.8 kb

2. The PCR reaction uses the following amplification cycles:

1) 94°C for 10 min.

<u>2)</u> 5 cycles:	<u>3)</u> 5 cycles:	<u>4)</u> 25 cycles:
95°C - 30 sec	95°C - 30 sec	95°C - 30 sec
61°C - 1 min	59°C - 1 min	51°C - 1 min
73°C - 5 min	75°C - 5 min	73°C - 5 min

15 5) 72°C for 8 min. The reactions are terminated by chilling to 4°C (hold).

3. The products are analyzed by electrophoresis- in a 1% agarose gel, comparing to an aliquot of the unlabelled probe starting material.

4. The amount of DIG-labeled probe is determined as follows:

Make serial dilutions of the diluted control DNA in dilution buffer (TE: 10 mM Tris and 1 mM EDTA, pH 8) as shown in the following table:

DIG-labeled control DNA starting conc.	Stepwise Dilution	Final Conc. (Dilution Name)
5 ng/ $\mu$ l	1 $\mu$ l in 49 $\mu$ l TE	100 pg/ $\mu$ l (A)
100 pg/ $\mu$ l (A)	25 $\mu$ l in 25 $\mu$ l TE	50 pg/ $\mu$ l (B)
50 pg/ $\mu$ l (B)	25 $\mu$ l in 25 $\mu$ l TE	25 pg/ $\mu$ l (C)
25 pg/ $\mu$ l (C)	20 $\mu$ l in 30 $\mu$ l TE	10 pg/ $\mu$ l (D)

5

- Serial deletions of a DIG-labeled standard DNA ranging from 100 pg to 10 pg are spotted onto a positively charged nylon membrane, marking the membrane lightly with a pencil to identify each dilution.
- Serial dilutions (e.g., 1:50, 1:2500, 1:10,000) of the newly labeled DNA probe are spotted.
- The membrane is fixed by UV crosslinking.
- The membrane is wetted with a small amount of maleate buffer and then incubated in 1% blocking solution for 15 min at room temp.
- The labeled DNA is then detected using alkaline phosphatase conjugated anti-DIG antibody (Boehringer Mannheim, Indianapolis, IN, cat. no. 1093274) and an NBT substrate according to the manufacturer's instruction.
- Spot intensities of the control and experimental dilutions are then compared to estimate the concentration of the PCR-DIG-labeled probe.

10

15

**D. Prehybridization and Hybridization of Southern Blots**Solutions:

100% Formamide purchased from Gibco

20X SSC (1X = 0.15 M NaCl, 0.015 M Na<sub>3</sub>citrate)5 per L: 175 g NaCl  
87.5 g Na<sub>3</sub>citrate·2H<sub>2</sub>O

20% Sarkosyl (N-lauroyl-sarcosine)

20% SDS (sodium dodecyl sulphate)

10 10% Blocking Reagent: In 80 ml deionized distilled water, dissolve 1.16 g maleic acid. Next, add NaOH to adjust the pH to 7.5. Add 10 g of the blocking reagent powder. Heat to 60°C while stirring to dissolve the powder. Adjust the volume to 100 ml with water. Stir and sterilize.

## Prehybridization Mix:

Final Concentration	Components	Volume (per 100 ml)	Stock
50%	Formamide	50 ml	100%
5X	SSC	25 ml	20X
0.1%	Sarkosyl	0.5 ml	20%
0.02%	SDS	0.1 ml	20%
2%	Blocking Reagent	20 ml	10%
	Water	4.4 ml	

General Procedures:

15 1. Place the blot in a heat-sealable plastic bag and add an appropriate volume of prehybridization solution (30 ml/100cm<sup>2</sup>) at room temperature. Seal the bag with a heat sealer, avoiding bubbles as much as possible. Lay down the bags in a large plastic tray (one tray can accommodate at least 4–5 bags). Ensure that the bags are lying flat in the tray so that the prehybridization solution is evenly distributed

throughout the bag. Incubate the blot for at least 2 hours with gentle agitation using a waver shaker.

2. Denature DIG-labeled DNA probe by incubating for 10 min. at 98°C using the PCR machine and immediately cool it to 4°C.

5 3. Add probe to prehybridization solution (25 ng/ml; 30 ml = 750 ng total probe) and mix well but avoid foaming. Bubbles may lead to background.

4. Pour off the prehybridization solution from the hybridization bags and add new prehybridization and probe solution mixture to the bags containing the membrane.

5. Incubate with gentle agitation for at least 16 hours.

10 6. Proceed to medium stringency post-hybridization wash:

Three times for 20 min. each with gentle agitation using 1X SSC, 1% SDS at 60°C.

All wash solutions must be prewarmed to 60°C. Use about 100 ml of wash solution per membrane.

15 To avoid background keep the membranes fully submerged to avoid drying in spots; agitate sufficiently to avoid having membranes stick to one another.

7. After the wash, proceed to immunological detection and CSPD development.

#### **E. Procedure for Immunological Detection with CSPD**

##### Solutions:

Buffer 1: Maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl; 20 adjusted to pH 7.5 with NaOH)

Washing buffer: Maleic acid buffer with 0.3% (v/v) Tween 20.

5

Blocking stock solution <sup>94</sup>  
10% blocking reagent in buffer 1. Dissolve (10X concentration): blocking reagent powder (Boehringer Mannheim, Indianapolis, IN, cat. no. 1096176) by constantly stirring on a 65°C heating block or heat in a microwave, autoclave and store at 4°C.

Buffer 2

(1X blocking solution): Dilute the stock solution 1:10 in Buffer 1.

Detection buffer: 0.1 M Tris, 0.1 M NaCl, pH 9.5

Procedure:

10 1. After the post-hybridization wash the blots are briefly rinsed (1-5 min.) in the maleate washing buffer with gentle shaking.

2. Then the membranes are incubated for 30 min. in Buffer 2 with gentle shaking.

3. Anti-DIG-AP conjugate (Boehringer Mannheim, Indianapolis, IN, cat. no. 1093274) at 75 mU/ml (1:10,000) in Buffer 2 is used for detection. 75 ml of solution can be used for 3 blots.

15 4. The membrane is incubated for 30 min. in the antibody solution with gentle shaking.

5. The membrane are washed twice in washing buffer with gentle shaking. About 250 mls is used per wash for 3 blots.

6. The blots are equilibrated for 2-5 min in 60 ml detection buffer.

20 7. Dilute CSPD (1:200) in detection buffer. (This can be prepared ahead of time and stored in the dark at 4°C).

The following steps must be done individually. Bags (one for detection and one for exposure) are generally cut and ready before doing the following steps.

8. The blot is carefully removed from the detection buffer and excess liquid removed without drying the membrane. The blot is immediately placed in a bag and 1.5 ml of CSPD solution is added. The CSPD solution can be spread over the membrane. Bubbles present at the edge and on the surface of the blot are typically removed by gentle rubbing. The membrane is incubated for 5 min. in CSPD solution.

5

9. Excess liquid is removed and the membrane is blotted briefly (DNA side up) on Whatman 3MM paper. Do not let the membrane dry completely.

10. Seal the damp membrane in a hybridization bag and incubate for 10 min at 37°C to enhance the luminescent reaction.

10 11. Expose for 2 hours at room temperature to X-ray film. Multiple exposures can be taken. Luminescence continues for at least 24 hours and signal intensity increases during the first hours.

**Example 3: Transformation of Carrot Cells**

Transformation of plant cells can be accomplished by a number of methods, as described above. Similarly, a number of plant genera can be regenerated from tissue culture following transformation. Transformation and regeneration of carrot cells as described herein is illustrative.

Single cell suspension cultures of carrot (*Daucus carota*) cells are established from hypocotyls of cultivar Early Nantes in B<sub>5</sub> growth medium (O.L. Gamborg et al., *Plant Physiol.* 45:372 (1970)) plus 2,4-D and 15 mM CaCl<sub>2</sub> (B<sub>5</sub>-44 medium) by methods known in the art. The suspension cultures are subcultured by adding 10 ml of the suspension culture to 40 ml of B<sub>5</sub>-44 medium in 250 ml flasks every 7 days and are maintained in a shaker at 150 rpm at 27 °C in the dark.

The suspension culture cells are transformed with exogenous DNA as described by Z. Chen et al. *Plant Mol. Bio.* 36:163 (1998). Briefly, 4-days post-subculture cells are incubated with cell wall digestion solution containing 0.4 M sorbitol, 2% driselase, 5mM MES (2-[N-Morpholino] ethanesulfonic acid) pH 5.0 for 5 hours. The digested cells are pelleted gently at 60 xg for 5 min. and washed twice in W5 solution containing 154 mM NaCl, 5 mM KCl, 125 mM CaCl<sub>2</sub> and 5mM glucose, pH 6.0. The protoplasts are

suspended in MC solution containing 5 mM MES, 20 mM CaCl<sub>2</sub>, 0.5 M mannitol, pH 5.7 and the protoplast density is adjusted to about  $4 \times 10^6$  protoplasts per ml.

15-60 µg of plasmid DNA is mixed with 0.9 ml of protoplasts. The resulting suspension is mixed with 40% polyethylene glycol (MW 8000, PEG 8000), by gentle 5 inversion a few times at room temperature for 5 to 25 min. Protoplast culture medium known in the art is added into the PEG-DNA-protoplast mixture. Protoplasts are incubated in the culture medium for 24 hour to 5 days and cell extracts can be used for assay of transient expression of the introduced gene. Alternatively, transformed cells can be used to produce transgenic callus, which in turn can be used to produce transgenic plants, by 10 methods known in the art. See, for example, Nomura and Komamine, *Plt. Phys.* 79:988-991 (1985), *Identification and Isolation of Single Cells that Produce Somatic Embryos in Carrot Suspension Cultures*. -

Additional *E. coli* Library deposits *E. coli*LibA021800 and *E. coli*Lib060700 were made at the American Type Culture Collection in Manassas, Virginia, USA on February 23, 15 2000 and June 8, 2000 respectively to meet the requirements of Budapest Treaty for the international recognition of the deposit of microorganisms. These deposits were assigned ATCC accession no. PTA-1411 and PTA-2007 respectively.

The invention being thus described, it will be apparent to one of ordinary skill in the art that various modifications of the materials and methods for practicing the invention can 20 be made. Such modifications are to be considered within the scope of the invention as defined by the following claims.

Each of the references from the patent and periodical literature cited herein is hereby expressly incorporated in its entirety by such citation.